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(57) Abstract

The invention relates to a factor which has activity in protecting a cell against the bacteriolytic enzyme, zoocin A. Nucleic acid which encodes the factor is useful in transforming GRAS organisms to be able to produce zoocin A without vulnerability to the activity of the enzyme itself. The resulting organisms can then be used in antibacterial compositions (particularly foodstuffs) against a range of bacteria, including *S. mutans, S. sobrinus* and *S. pyogenes*.

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ZOOCIN A IMMUNITY FACTOR

TECHNICAL FIELD

The invention relates to a factor which has activity in protecting a cell producing zoocin A, to the gene encoding that factor, to vectors and organisms containing the gene and the use of such organisms as anti-bacterial agents.

BACKGROUND ART

Since the dawn of microbiology it has been observed that the growth of some strains of bacteria can interfere with the growth of other potentially harmful bacteria growing in the same medium. We now know that these inhibitory reactions are mediated by a range of metabolic and protein products produced by many different strains of bacteria. The "classical" antibiotics such as streptomycin and penicillin are metabolic (enzyme synthesized) products and their use in the prevention and treatment of disease is now well established. In contrast, industrial and medical use of proteinaceous (ribosomally synthesized) inhibitory substances has been much more limited. Recently however, this situation has changed and in 1988 nisin was granted GRAS (Generally recognized as safe) status by the U.S. Food and Drug Administration (Federal Register 1988) in recognition of the fact that nisin was produced by *Lactococcus lactis* strains naturally associated with certain foods during processing and that it has no apparent adverse effects when ingested.

Zoocin A is a unique domain-structured bacteriolytic enzyme produced by *Streptococcus equi* subsp. *zooepidemicus* 4881, which specifically attacks the cell walls of some closely related streptococcal species including the principal causative agents of group A streptococcal sore throat and dental caries respectively (Simmonds *et al* (1995); Simmonds *et al* (1996)). It was shown that zoocin A could suppress the growth of *S. mutans* in a triple species plaque model and that the initiation of the killing sequence occured very quickly. A 6.8 kb *Eco*R I fragment containing the gene encoding zoocin A (*zooA*) was cloned into *Escherichia coli* using the pBluescript® II SK(+) phagemid vector and the sequence of *zooA* determined (Simmonds *et al* (1997)). The N-terminal catalytic domain of zoocin A has a high degree of homology with the N-terminal catalytic domain of a similar bacteriolytic

enzyme lysostaphin, produced by *Staphylococcus simulans* biovar *staphylolyticus*, which specifically attacks the cell walls of other staphylococcal species. The C-terminal substrate-binding domain of lysostaphin is known to have a high degree of homology to at least one other staphylococcal cell wall binding enzyme, a *Staph. aureus* amidase. By contrast, the substrate-binding domain of zoocin A has homology to no other known sequence. Both enzymes appear to lyse cell walls by cleaving the peptide cross-links within the peptidoglycan (Simmonds *et al* (1996)). The bacteriocidal nature of their mode of action and the high degree of species and strain specificity exhibited by these enzymes are characteristics of that group of proteinaceous inhibitory agents referred to as bacteriocin-like inhibitory substances (BLIS).

Zoocin A targets only a very limited range of bacteria, restricted to some species of *Streptococcus* only. This species-specific anti-bacterial action is useful. For example, it is active against two groups of medically significant human pathogens and at least one significant animal pathogen.

S. mutans and *S. sobrinus* are two of twenty or more species of bacteria present in dental plaque. Although not numerically dominant, these two species are considered to be the major aetiological agents of dental caries and their suppression in the oral cavity has been shown to reduce caries incidence (Loesche (1976); Loesche *et al* (1989)). Group A streptococci (GAS) infect via the upper respiratory tract where the tonsillar region in particular is believed to be the primary site of colonization. GAS carriage in humans is relatively common and GAS pharyngitis left untreated can progress to more serious disease including rheumatic fever and nephritis (Bronze and Dale (1996)). Vaccines are not available to prevent these infections and although it has been shown that these groups of microorganisms can be suppressed in the oral cavity by administration of antibacterial agents such as chlorhexidine (Loesche (1976)), polyvalent cations (Jones *et al* (1988)) and classical antibiotics (Loesche *et al* (1989)), the broad spectrum nature of these agents means that many commensal organisms are also suppressed, a condition which is known to pre-dispose the patient to superinfection by resistant microoganisms including gram-negative bacteria and yeasts. In each case the prolonged and widespread use of these agents has not been considered acceptable (Marsh (1991)). In contrast, zoocin A, while having significant bacteriocidal activity against these groups of

microorganisms has little or no activity against many other plaque species such as *S. oralis* (Simmonds *et al* (1996)), *S. sanguis* or non-streptococcal species (Simmonds *et al* (1995)), or against the major groups colonizing the mucosal surfaces of the oral cavity such as *S. salivarius* (Simmonds *et al* (1995)). Therefore, administration of zoocin A to the oral cavity is unlikely to result in the complications seen with the previously mentioned broad spectrum anti-microbial agents, yet should lead to a decrease in the incidence of dental caries and carriage of GAS.

Before zoocin A can be used for its desirable anti-bacterial properties, there is a need for it to be provided in a form that can be administered to a human or an animal safely. For many antibiotics this is achieved by batch fermentation of the organism producing the antibiotic and purifying the antibiotic molecule and adding it to a suitable carrier. This method would be very expensive for zoocin A which has a molecular weight of 28,000. For that reason, the more commercially attractive option is to produce the zoocin A *in situ* in a naturally fermented food such as yoghurt.

However, zoocin A is produced by *S. equi* subsp. *zooepidemicus*, a recognized animal and occasional human pathogen. Serious human disease has been shown to result from the ingestion of *S. equi* subsp. *zooepidemicus* contaminated unpasteurized milk (Francis *et al* (1993)). Therefore, use of the natural producer organism to incorporate zoocin A in a food product as part of a food fermentation process is unlikely to be acceptable, but one solution would be to move the genes required for zoocin A production from the natural host to an organism suitable for use in food fermentation processes. However, this approach presents some difficulties when zoocin A is lethal to the genetically transformed organism.

One solution to these difficulties is to render the organism which is to express zoocin A resistant (immune) to the activity of this enzyme. This solution requires a factor to be identified which protects otherwise susceptible organisms against zoocin A activity.

The applicants have now identified such a factor, which is generally referred to hereinafter as zoocin A immunity factor. It is towards this factor and to its use that the present invention is broadly directed.

SUMMARY OF THE INVENTION

In one aspect, the invention provides zoocin A immunity factor, which is a protein which is capable of protecting a host cell expressing zoocin A against the potentially damaging activity of zoocin A.

In a further aspect the invention provides an isolated DNA molecule which has a nucleotide sequence which encodes zoocin A immunity factor (*zif*).

Preferably the DNA molecule is selected from the group comprising molecules having one or more of: the *zif* sequence shown in Figure 3, a sequence comprising that sequence, a sequence comprising a part of that sequence active in protecting an organism from zoocin A, a sequence encoding the same protein as the *zif* sequence of Figure 3 but differing in nucleic acid sequence by virtue of degeneracy of the genetic code and a sequence which is a functionally equivalent variant of the *zif* sequence shown in Figure 3.

In still a further aspect of the invention, there is provided a vector comprising the *zif* encoding molecule defined above, optionally together with a gene encoding the zoocin A active protein or variant defined above.

In yet a further aspect, the invention provides a non-pathogenic organism containing the *zif* encoding molecule defined above, optionally together with a gene encoding a polypeptide sequence selected from the sequence for zoocin A or a functionally equivalent variant of that sequence.

Preferably, the organism is a food-grade organism.

As another aspect of the invention, there is provided an antibacterial composition comprising a non-pathogenic organism as defined above.

Preferably, the composition is suitable for ingestion, particularly human ingestion, and is a foodstuff, nutriceutical or confectionery.

In yet a further aspect, the invention provides a method of preventing or inhibiting the growth of undesirable organisms susceptible to zoocin A which comprises the step of contacting said organisms or the environment thereof with a composition as defined above.

Preferably, the organisms inhibited are *S. mutans*, *S. sobrinus* or *S. pyogenes* and the composition is administered to the oral cavity of a patient.

Other aspects of the invention will be apparent from the description provided, and from the claims.

DESCRIPTION OF THE DRAWINGS

While the invention is broadly as defined above, it further includes embodiments of which the following description provides examples. It will also be better understood with reference to the accompanying drawings in which:

Figure 1 shows a map of pBluescript® II SK(+) phagemid vector and pVA838.

Figure 2 is a restriction map of PDN488L showing ORFs and subclones. The nucleotides are numbered from the first nucleotide of the *Eco*R I restriction site located proximal to the *Sac* I restriction site in the pBluescript® II SK(+) phagemid vector *Sac* I - *Kpn* I MCS of pDN488L. The translation is in the direction indicated by the bold arrows.

Figure 3 shows the DNA sequence of 6.8 kb base *Eco*R I fragment showing the nucleotide and amino acid sequences for both *zooA* and *zif*. It will be appreciated that the strand of nucleic acid coding for *zif* is complementary to the non-coding strand shown expressly in Figure 3.

DESCRIPTION OF THE INVENTION

The focus of the invention is on the applicants identification of the gene encoding zoocin A immunity factor (*zif*). This gene is capable of protecting cells which express zoocin A against the effects of that enzyme.

The *zif* gene has been identified from *S. equi subsp. zooepidemicus* 4881 and has the sequence given in Figure 3. This sequence is of the non-coding strand, with the coding strand being complementary. The sequence of the coding strand is recited as SEQ ID NO. 2.

However, it will be appreciated that the sequence need not always be that shown in Figure 3 but can instead be a functionally-equivalent variant of that sequence. Such variants are in no way intended to be excluded and the resultant molecules are referred to herein as "*zif*-like genes".

The amino acid sequence of *zif* (which is coded for by the nucleotides of the coding strand) is also shown in Figure 3. Again, variations are possible while retaining functional equivalency.

The phase "functionally equivalent variants" recognises that it is possible to vary the amino acid/nucleotide sequence of a protein while retaining substantially equivalent functionality. For example, a protein can be considered a functional equivalent of another protein for a specific function if the equivalent protein is immunologically cross-reactive with and has at least substantially the same function as, the original protein. The equivalent can be, for example, a fragment of the protein, a fusion of the protein with another protein or carrier, or a fusion of a fragment which additional amino acids. For example, it is possible to substitute amino acids in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids normally held to be equivalent are:

(a) Ala, Ser, Thr, Pro, Gly;

(b) Asn, Asp, Glu, Gln;

(c) His, Arg, Lys;

(d) Met, Leu, Ile, Val; and

(e) Phe, Tyr, Trp.

Equally, DNA sequences encoding a particular produce can vary significantly simply due to the degeneracy of the nucleic acid code.

The probability of one sequence being functionally equivalent to another can be measured by the computer algorithms BLASTP (Altschul, S. F. *et al* (1990)) and FASTA (Pearson, W. R. *et al* (1988)) for proteins and DNA respectively.

The *zif* gene or *zif*-like gene of the invention can be inserted into organisms which are to be transformed with the *zooA* gene (which encodes zoocin A) so that a recipient organism which is zoocin A sensitive is protected by expression of the *zif* gene. The action of *zif* in protecting a zoocin A producer cell from the otherwise lethal action of its own product is believed to involve the modification of the cells peptidoglycan cross-links to a chemical form non-hydrolysed by zoocin A.

Organisms which may be usefully transformed with the *zif* gene include any food-acceptable or pharmaceutically acceptable non-pathogenic organism. When the gene is inserted into zoocin A susceptible organisms, these organisms can be subsequently or simultaneously transformed with *zooA* in a manner which allows production of zoocin A. The *zif* gene protects the transformed organism from the lethal effects of zoocin A produced.

It will of course be appreciated that the terms "transformed" or "transformation" are used herein in their broadest possible sense. While normally a recombinant transformation process will be employed, any so-called "natural transfer" approach can also be used. "Natural transfer" approaches involve the placement of an organism including DNA encoding both *zif* and zoocin A in the proximity of the organism to which the DNA is to be transferred, and allowing exchange to occur naturally.

Both recombinant and natural transfer of DNA from one host organism to another is now routine in the art. It will therefore be appreciated that any conventional approach can be employed, so long as the desired transformation occurs.

It will however be more usual to effect transformation by recombinant means. This is the preferred approach taken for this invention and normally will involve the use of transformation vectors/gene constructs.

While it is conceivable that separate vectors/constructs could be employed to separately transfer the *zif* and zoo A genes to a recipient organism, it would be more usual for both genes to be contained in the same vector/construct.

The vector pSB1131 is a preferred vector for this purpose.

Preferred non-pathogenic organisms for use in the invention include yeasts and bacteria. In particular, organisms having a genus selected from non-pathogenic strains of streptococcus are particularly useful. Especially preferred are non-pathogenic strains of *Streptococcus gordonii*.

Organisms transformed with the gene of the invention may be used as preservatives in processed cheese, various pasteurised dairy products, canned vegetables, hot baked flour products and pasteurised liquid egg. They may also be used in preservation of naturally fermented foods such as beer, wine, yoghurt and cheeses.

The transformed organisms and/or extracts of the organisms may also be used to prepare pharmaceutical compositions for use topically to prevent establishment of infectious diseases of humans and animals. Such topical compositions are useful in treatment of skin conditions, such as ulcers, in which streptococci are significant pathogens and where poor blood supply limits the effectiveness of systemically administered antibiotics.

Group C streptococci are serious animal pathogens, particularly of horses and are responsible for considerable economic loss to the bloodstock industry. As with GAS in humans, the primary route of infection for these organisms is believed to be the respiratory tract and it is contemplated that the incorporation of organisms according to the invention which express zoocin A with animal feeds may reduce colonization rates in these animals, and hence the rate of serious disease.

It is however presently preferred that the transformed organisms and/or their zoocin A-containing culture fluid be included in a composition intended for human ingestion (such as a foodstuff, nutriceutical or confectionery). This is particularly the case where the intention is to treat or prevent problems associated with the organisms *S. mutans* and/or *S. sobrinus*. These organisms inhabit the oral cavity and, as stated previously, are considered to be the major aetiological agents of dental caries. Their suppression in the oral cavity reduces the incidence of dental caries.

Further, this is particularly the case where the intention is to treat or prevent problems associated with *S. pyogenes*. These organisms colonise the tonsillar region of the oral cavity and, as stated previously, are the major aetiological agents of GAS associated disease.

Foodstuffs such as processed cheeses and yoghurts are particularly appropriate for such applications. Confectioneries such as wine gums and chewing gums are also contemplated.

The transformed organism of the invention may be admixed with food products, confectioneries and pharmaceutical carriers by conventional means. For fermented products such as yoghurts, conventional methods may also be used including the step of adding the transformed microorganism at the time of culturing the product. Preferably the transformed microorganism is of the same species as conventionally used for the preparation of the fermented product thus allowing the preparation of the zoocin A and the fermented product to occur simultaneously.

The invention will now be described with reference to the following non-limiting examples.

EXAMPLE 1

Materials and Methods.

i) Bacterial strains and plasmids.

Stock cultures of all strains were stored in skim milk at -70°C. Strains in regular use were maintained as plate cultures and subcultured every two weeks. *E. coli* DH5αF' (Woodcock *et al* (1989), Raleigh *et al* (1989)) was grown routinely at 37°C in air and *S. equi* subsp. *zooepidemicus* 4881 (Schofield and Tagg (1983)) and *S.gordonii* DL1 (Macrina *et al* (1982)) in 5% CO_2 in air atmosphere at 37°C.

E. coli DH5αF' was routinely cultured in 2xYT medium (16 g bacto-tryptone (Difco Laboratories, Detroit, MI, USA), 10 g bacto-yeast extract (Difco), and 5 g NaCl (Riedel-de Haën AG, Seeize, Germany) to one litre of distilled water, purified with a Milli-Q system (Millipore Inc., France) (MQ water), Luria-Bertani (LB) medium (10 g bacto-tryptone (Difco), 5 g bacto-yeast extract (Difco), and 10 g NaCl (Riedel-de Haën AG) to one litre of MQ water) or on LB agar (LBA) plates. LBA was prepared by supplementing LB medium with 1.5% bacto-agar (Difco). Plates containing antibiotics were prepared by supplementing LBA with either 100 mg/ml ampicillin (LBA+Ap), 250 mg/ml erythromycin (LBA+Em250), 500 mg/ml erythromycin (LBA+Em500) or 25 mg/ml chloramphenicol (LBA+Cm). All antibiotics were manufactured by Sigma (Sigma Chemical Co., St. Louis, MO, USA). LBA containing antibiotics was stored at 4°C for periods of up to two weeks.

Streptococcus gordonii DL1 strains were routinely cultured in Todd Hewitt broth (THB) (Difco), on Columbia Agar Base (CAB) (GIBCO BRL, Life Tec. Ltd., Paisly UK) plates or on blood agar (BA) (CAB supplemented with 5% whole human blood (Dunedin Public Hospital, Dunedin, NZ)). Antibiotic containing agar plates were prepared by supplementing CAB with 10 mg/ml erythromycin (CAB+Em). Prior to transformation *S. gordonii* DL1 were grown in Brain Heart Infusion (BHI) (Difco) supplemented with 0.5% bacto-yeast extract (Difco), 1% membrane filtered horse serum (GIBCO BRL) and 0.1% glucose (Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany) (BHS broth). CAB containing antibiotics was stored at 4°C for periods of up to two weeks.

Bacterial strains and their plasmids used in this study are described in Table 1. Maps of pBluescript® II SK(+) phagemid vector (Stratagene, La Jolla, CA, USA) and pVA838 (Macrina *et al* (1982)) are given in Figure 1.

ii) Genetic manipulations.

Restriction enzyme digestion, ligation, and electrophoresis procedures.

Unless otherwise stated, cloning methods were carried out as previously described (Sambrook *et al* (1989)). Restriction digests were performed according to the manufacturers instructions; *Eco*R I, *Pst* I, *Hind* III, *Xba* I and *Pvu* II (Boehringer Mannheim Gmbh, Mannheim, Germany); *Cla*I and *Eco*RV (Amersham International plc, Amersham, UK); and *Sma* I (New England Biolabs, Beverly, MA, USA). Calf Intestinal Phosphatase (CIP) (New England Biolabs) was used to treat vector digests prior to ligation as per the manufacturers instructions. Ligations were performed at temperatures between 12°C and 15°C overnight using T4 DNA ligase (Boehringer Mannheim Gmbh) as per the manufacturers instructions. Prior to use in transformations, ligation mixtures were ethanol precipitated with 1 µl glycogen (Boehringer Mannheim Gmbh) and resuspended in 10 µl Milli-Q water.

Unless otherwise stated, gel electrophoresis was performed using 1% agarose (Sigma) gels prepared and run with Tris-acetate EDTA (TAE) buffer (per litre: 4.84 g Tris base (Serva), 1.142 ml glacial acetic acid (Rhône-Poulenc Chemicals Ltd., Bristol, UK), and 0.8 ml 0.5 M ethylenediaminetetra-acetate (BDH Laboratory Supplies, Poole, UK) (EDTA) at 75 - 100 V. Electrophoresis was performed using a Pharmacia Electrophoresis Constant Power Supply ECPS 2000/300 (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and gel electrophoresis apparatus including a range of submarine gel tanks: 20 cm x 24 cm Model H4 (Betheseda Research Laboratories, Gaithersburg, MD, USA), 11 cm x 14 cm HORIZON 11*14 (GIBCO BRL), 8 cm x 6 cm minigel tank (Bio-rad).

***E. coli* DH5αF' electro-transformation.**

Unless otherwise stated, preparation of electo-competent *E. coli* DH5αF' cells and electro-transformation of electro-competent *E. coli* DH5αF' cells was performed as previously described (Dower (1988)). *E. coli* DH5αF' electro-transformations were performed with a Biotechnologies and Experimental Research Inc. (BTX) BTX® *E.*

coli TransPorator™ (BTX, SanDiego, CA, USA), a Pharmacia LKB 2197 Power Supply (Pharmacia LKB, Broma, Sweden), and 0.1 cm electrode gap Gene Pulser™ Cuvettes (Bio-rad Laboratories, Hercules, CA, USA). 40 µl aliquots of *E. coli* DH5αF' electro-competent cells were maintained at -70°C until required. Following electro-poration, 1 ml of 2xYT broth was immediately added to the transformation mixture and the cells resuspended and transferred to a glass vial. Resuspended cells were incubated at 37°C with shaking at 200 rpm for 1 hour to enable the plasmid encoded antibiotic resistance genes to be expressed. Dilutions of the mixture were spread plated on appropriate antibiotic-containing media and incubated at 37°C overnight.

Characterisation of *E. coli* DH5αF' transformants carrying recombinant pBluescript® II SK(+) phagemid vectors.

Colonies growing on LBA+Ap were patched with a sterile toothpick onto LBA+Ap screening plates spread with 4 µl of 200 mg/ml Isopropyl-b-D-thiogalactoside (IPTG) (Boehringer Mannheim GmbH) and 40 µl of 20 mg/ml 5'-Bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal) (Boehringer Mannheim GmbH). After overnight incubation *E. coli* DH5αF' transformants containing Bluescript® II SK(+) phagemid vectors (Stratagene) (Alting-Mees *et al*, 1989; Short *et al*, 1988) with inserts were identified as white patches amongst a background of blue patches. A small amount of culture was picked from each white patch with a toothpick and resuspended in 25 µl of cracking solution (In one ml: 835 µl MQ water, 100 µl glycerol (BDH), 25 µl 20% Sodium Dodecyl Sulphate (SDS) (BDH), 25 µl 2 M NaOH (BDH), 10 µl 0.5 M EDTA (BDH) and 5 µl 2% bromocresol green (J.T. Baker Co., Phillipsburg, NJ, USA)) and incubated at 65°C for 30 minutes. After incubation each sample was carefully loaded into dry wells in an agarose gel and electrophoresed at 40 V for approximately 15 minutes until each sample had completely entered the gel. TAE buffer was then added to cover the gel and electrophoresis continued at 75 - 100 V until completion. DNA bands were visualized by staining the gel for 10 minutes in 0.5 µg/ml ethidium bromide (Sigma) solution. Supercoiled plasmids were clearly visible after ethidium bromide staining. Recombinants were initially characterized by comparing their plasmid size with the plasmid size of supercoiled pBluescript® II SK(+) phagemid vector carrying no insert.

E. coli DH5αF' transformants yielding appropriately sized plasmids were used to inoculate 2.5 ml 2xYT broth supplemented with 100 μg/ml ampicillin. Following overnight incubation at 37°C plasmid DNA was extracted from 1.5 ml of each culture using the Quantum prep™ plasmid miniprep kit (miniprep) (Bio-rad) and the plasmid DNA eluted from the miniprep matrix in 100 ml of MQ water according to the manufacturers instructions. The eluted DNA was stored at -20°C. The remaining culture was centrifuged and the pellet resuspended in 10% skim milk and stored at -70°C.

Those transformants carrying pBluescript® II SK(+) phagemid vector with an insert were characterized by restriction digestion of miniprep plasmid DNA. Plasmid DNA was digested with restriction enzymes chosen to linearise the plasmid. *Eco*R I was used to linearise plasmid DNA from pSB1006, pSB1291, pSB1205, and pSB1014 transformants. *Sac* I was used to linearise plasmid DNA from pSB10313 and pSB1047 transformants, *Hind* III to linearise plasmid DNA from pSB1083 transformants, and *Pst* I to linearise plasmid DNA from pSB961 and pSB981 transformants. The digested plasmid DNA was electrophoresed and the size of the plasmid determined relative to known DNA sizing standards (either *Pst* I or *Hind* III digested l DNA (New England Biolabs)). DNA bands were visualized by staining the gel for 10 minutes in 0.5 μg/ml ethidium bromide (Sigma) solution. The size estimate obtained for each plasmid was compared with the predicted size determined from the previously published restriction map of pDN488L (Simmonds *et al* (1997)).

Characterisation of *E. coli* DH5αF' transformants carrying recombinant pVA838 vectors.

E. coli DH5αF' colonies visible on LBA+Em250 after 12 - 16 hours incubation were streaked onto LBA+Em500 and LBA+Cm plates and incubated overnight at 37°C. Transformants able to grow overnight on LBA+Em500 but not on LBA+Cm were initially characterized as previously described (Characterisation of *E. coli* DH5αF' transformants carrying recombinant pBluescript® II SK(+) phagemid vectors) and the size of their supercoiled plasmids compared with the size of supercoiled pVA838 (Macrina *et al* (1982)).

E. coli DH5αF' isolates identified as carrying plasmids of the appropriate size were grown overnight at 37°C in 5 ml 2xYT broth supplemented with 500 μg/ml Em. Plasmid DNA was extracted from 3 ml of each culture using the Quantum prep™ plasmid miniprep kit (Bio-rad) and the plasmid DNA eluted from the miniprep matrix in 100 ml of MQ water according to the manufacturers instructions. The eluted DNA was stored at -20°C. The remaining culture was centrifuged and the pellet resuspended in 10% skim milk and stored at -70°C.

Transformants carrying pVA838 vector with an insert were characterized by restriction digestion of miniprep plasmid DNA essentially as described previously (Characterisation of *E. coli* DH5αF' transformants carrying recombinant pBluescript® II SK(+) phagemid vectors). *Eco* R I was used to linearise plasmid DNA from pSB1847 transformants whereas *Eco* R I digestion of plasmid DNA from pSB1311 transformants yielded two fragments (ie. 6.8 kb insert and 9.2 kb vector).

Construction of subclones using pBluescript® II SK(+) phagemid vector.

Plasmids were constructed using a subcloning strategy based on the previously published restriction map of pDN488L (Simmonds *et al* (1997)). The cloning of pDN488L, pDN2.2, and pDN0.8 has been previously described. Unless otherwise stated the following method was used to construct all pBluescript® II SK(+) phagemid vector subclones.

At least 1 μg pBluescript® II SK(+) phagemid vector miniprep DNA was digested with the appropriate restriction enzyme(s), treated with CIP and electrophoresed. Unless otherwise stated, at least 1 μg of the appropriate parent plasmid miniprep DNA was digested with the appropriate restriction enzyme(s), treated with CIP and electrophoresed. Bands corresponding to the 2.9 kb linearised pBluescript® II SK(+) phagemid vector, and the desired insert fragment (Table 1) were extracted from the gel using a Prep-A-Gene™ DNA purification kit (Bio-rad), eluted with 30 μl MQ water according to the manufacturers instructions and ligated. Following ligation of the vector and insert, electro-competent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying recombinant pBluescript® II SK(+) phagemid vectors).

An alternative method was used to construct pSB1006, pSB1014, and pSB1025. A restriction enzyme was chosen that cut once within the 6.8 kb insert of pDN488L and once within the pDN488L multi-cloning site (MCS). Restriction digestion produced two fragments, one corresponded to linearised pBluescript® II SK(+) phagemid vector incorporating a section of pDN488L, and the other corresponded to the remaining region of pDN488L and a short segment of the MCS. The digest was electrophoresed and the band corresponding to linearised pBluescript® II SK(+) phagemid vector incorporating pDN488L DNA was extracted from the gel using a Prep-A-Gene™ DNA purification kit (Bio-rad), eluted with 30 µl MQ water according to the manufacturers instructions and self-ligated. Following self-ligation, electro-competent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying pBluescript® II SK(+) phagemid vectors). pSB1083 was constructed similarly, differing in that the parent plasmid was pSB1014. pSB1047 was constructed similarly, differing in that the parent plasmid was pSB1006 and that two enzymes with unique but compatible restriction sites were used to digest pSB1006.

pSB961 was pBluescript® II SK(+) phagemid vector incorporating the 0.7 kb *Eco* RV - *Pst* I fragment of pDN2.2.

pSB981 was pBluescript® II SK(+) phagemid vector incorporating the 1.5 kb *Eco* RV - *Pst* I fragment of pDN2.2.

pSB1006 was pBluescript® II SK(+) phagemid vector incorporating the 3.7 kb *Cla* I - *Eco*R I fragment of pDN488L. A *Cla* I digestion of pDN488L was electrophoresed and the 6.6 kb band was extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

pSB1014 was pBluescript® II SK(+) phagemid vector incorporating the 3.1 kb *Hin*d III - *Eco*R I fragment of pDN488L. A *Hin*d III digestion of pDN488L was electrophoresed and the 6.0 kb band extracted from the gel and self-ligated as

described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

pSB1025 was pBluescript® II SK(+) phagemid vector incorporating the 3.4 kb *Eco* RV - *Eco*R I fragment of pDN488L. An *Eco* RV digestion of pDN488L was electrophoresed and the 6.3 kb band extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

pSB1083 was pBluescript® II SK(+) phagemid vector incorporating the 2.3 kb *Hind* III - *Xba* I fragment of pSB1014. A *Xba* I digestion of pSB1014 was electrophoresed and the 5.2 kb band extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

pSB10313 was pBluescript® II SK(+) phagemid vector incorporating the 0.8 kb *Xba* I - *Eco*R I fragment of pSB1014.

pSB1047 was pBluescript® II SK(+) phagemid vector incorporating the 0.2 kb *Cla* I - *Eco* RV fragment of pSB1006. An *Eco* RV/*Sma* I digestion of pSB1006 was electrophoresed and the 3.1 kb band extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

pSB1097 was pBluescript® II SK(+) phagemid vector incorporating the 0.3 kb *Hind* III - *Eco*R I fragment of pSB1025.

pSB1291 was pBluescript® II SK(+) phagemid vector incorporating the 4.0 kb *Pst* I - *Eco*R I fragment of pDN488L.

Construction of clones using pVA838 vector.

The following procedure was used to construct pSB1311 in *E. coli* DH5αF'. pVA838 miniprep DNA (at least 1 µg) was digested with *Eco*R I, treated with CIP and electrophoresed. pDN488L miniprep DNA (at least 1 µg) was digested with *Eco*R I,

treated with CIP and electrophoresed. Bands corresponding to the 9.2 kb *Eco*R I digested pVA838 vector and the 6.8 kb *Eco*R I digested pDN488L insert were extracted from the gel using the Prep-A-Gene™ DNA purification kit (Bio-rad) and eluted with 30 µl MQ water according to the manufacturers instructions. Following ligation of the vector and insert, electro-competent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying recombinant pVA838 vectors).

The following procedure was used to construct pSB1847 in *E. coli* DH5αF'. pVA838 miniprep DNA (at least 1 µg) was digested with *Eco*R I and *Pvu* II, treated with CIP and electrophoresed. pSB1291 miniprep DNA (at least 1 µg) was digested with *Eco*R I and *Sma* I and electrophoresed. Bands corresponding to the 8.9 kb *Eco*R I/*Pvu* II digested pVA838 vector and the 4 kb *Eco*RI/*Sma* I pSB1291 insert were extracted using the Bio-rad Gel Extraction Kit (Bio-rad) and eluted with 30 µl MQ water according to the manufacturers instructions. Following ligation of the vector and insert, electrocompetent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying recombinant pVA838 vectors).

Transformation of *S. gordonii* DL1 with pSB1311 and pSB1847.

S. gordonii DL1 was freshly subcultured on CAB prior to each transformation. 50 µl of an overnight culture of *S. gordonii* DL1 in BHS broth was used to inoculate 5 ml of pre-warmed BHS broth and the culture incubated (with a loosened cap) at 37°C in 5% CO_2 in air for 3 hours. 50 µl of this was used to inoculate 5 ml of pre-warmed BHS broth and the culture incubated (with a loosened cap) at 37°C in 5% CO_2 in air for a further one hour. After one hour the culture was dispensed in 0.8 ml volumes into glass vials and mixed with 10 - 50 µl (containing a minimum of 1 µg of DNA) of pSB1311 and pSB1847 miniprep DNA obtained from *E. coli* DH5αF' (pSB1311) and (pSB1847). Vials containing *S. gordonii* DL1 cells and pVA838 with no insert or *S.gordonii* DL1 cells and no DNA were included in each experiment as positive and negative controls respectively. Transformation mixtures were incubated for 3 - 4

hours at 37°C in 5% CO_2 in air before dilutions of each mixture were spread plated on CAB+Em and the plates incubated for 24 hours at 37°C in 5% CO_2 in air.

After incubation colonies were picked from the transformation plates, streaked onto CAB+Em and incubated overnight at 37°C in 5% CO_2 in air. Plasmid DNA was extracted from each isolate as previously described (Vriesema *et al*, 1996) and resuspended in 30 µl MQ water. *S. gordonii* DL1 plasmid DNA obtained in this way was characterized by restriction analysis as previously described (Characterisation of *E. coli* DH5αF' transformants carrying recombinant pVA838 vectors). Plasmid DNA extracted from *S. gordonii* DL1 (pSB1311) and (pSB1847) transformants was similarly compared with plasmid DNA extracted from *E. coli* DH5αF' (pSB1311) and (pSB1847) transformants respectively. The *E. coli* DH5αF' plasmid DNA used for comparison with the *S. gordonii* DL1 plasmid DNA originated from the same miniprep sample used in the respective *S. gordonii* DL1 transformation. Transformants were stored in 10% skim milk at -70°C.

iii) Phenotypic characterization of DL1 transformants.

Testing for BLIS production by deferred antagonism.

BLIS production was assessed using the deferred antagonism procedure (Tagg & Bannister (1979)). Briefly, a 1-cm wide streak of the test strain was inoculated diametrically across the surface of CAB plates using a cotton swab heavily charged with cells from a freshly grown THB culture. The inoculated plates were incubated at 37°C for 18 hour in air plus 5% CO_2 after which the visible growth was removed by scraping with the edge of a glass slide. The surface of the medium was sterilized by exposure to chloroform vapour for 30 minutes, aired for 30 minutes and the nine standard indicator strains (I1, *Micrococcus luteus;* I2, *S. pyogenes;* I3, *S. anginosus*; I4, *S. uberis*; I5, *S. pyogenes*; I6, *Lactococcus lactis* subsp. *lactis*; I7, *S. pyogenes*; I8, *S. pyogenes* and I9, *S. equisimilis*) (Tagg *et al*, 1979) inoculated from 18 hour THB cultures across the line of the original producer strain with use of cotton swabs. After incubation for 18 hours in 5% CO_2 at 37 °C the extent of inhibition of each indicator strain was recorded as: '-' for no inhibition and '+' if the zone was wider than each edge of the producer streak.

Testing for BLIS production by the surface spot method.

BLIS activity in liquid samples was quantitated using the surface spot method (SSM) described by Jack (1991). Briefly, a 20 µl droplet of the sample to be tested was spotted out on the surface of a CAB plate and left to soak into the agar plate. The plate surface was then sterilized by exposure to choloroform vapour for 30 minutes, aired for 30 minutes and standard indicator I2 (overnight culture in THB broth) swabbed evenly onto the surface of the plate. Following overnight incubation at 37°C for 18 hours in air plus 5% CO_2, the presence of inhibitory activity was visualized as a circular zone of inhibition in the I2 lawn at the site of droplet deposition. The titre of inhibitory activity in the samples were determined by making doubling dilutions of the test samples and plating out 20 ml drops of each dilution. The reciprocal of the highest doubling dilution at which inhibitory action was observed is given as the titre.

Testing for Zoocin A production.

S. gordonii DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) and (pSB1847) were tested for zoocin A production by the deferred antagonism method.

Testing for sensitivity to Zoocin A.

S. gordonii DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) and (pSB1847) were tested for sensitivity to zoocin A by both a modification of the deferred antagonism method, and a modification of the SSM. In the modified deferred antagonism method, the zoocin A producer strain, *S. equi* subsp. *zooepidemicus* 4881 was used as the test strain and *S. gordonii* DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) and (pSB1847) , standard indicators I1 and I2 and *S. equi* subsp. *zooepidemicus* 4881 used as the indicator strains. In the modified SSM, a partially purified preparation of zoocin A was diluted two-fold and 20 ml drops spotted onto the surface of CAB plates. The presence of inhibitory activity was visualized by swabbing onto the surface of each plate a lawn of either *S. gordonii* DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) or (pSB1847), standard indicator I1 or I2 or *S. equi* subsp. *zooepidemicus* 4881.

iv) Sequencing the regions flanking *zooA*.

Subcloning and primer selection.

Plasmid DNA used for double stranded DNA sequencing was obtained from *E. coli* DH5αF' or *E. coli* XL1 blue pBluescript® II SK(+) phagemid vector subclones by miniprep. *E. coli* DH5αF' and XL1 blue pBluescript® II SK(+) phagemid vector subclones have been previously described (See Figure 2 and section; Construction of subclones using pBluescript® II SK(+) phagemid vectors).

Table 2 contains a description of the primers used in this study. Universal M13 forward and reverse primers were synthesized by the Oligonucleotide Unit (Department of Biochemistry, University of Otago, Dunedin, NZ) and all other primers were synthesized by GIBCO BRL Custom Primers (GIBCO BRL). Universal M13 forward and reverse primers were used in sequencing reactions with pDN0.8, pSB961, pSB981, pSB1006, pSB1025, pSB10313, pSB1047, pSB1083 and pSB1291 plasmid DNA. SB108.3F2 and SB108.3R2 primers were designed from the sequence data obtained from sequencing pSB1083 using universal M13 forward and reverse primers respectively. Primers SB108.3F2 and SB108.3R2 were used in sequencing reactions with pSB1083 plasmid DNA. 6.8kbcontig1 to 6.8kbcontig12 primers were designed from contiguous sequence data obtained from sequencing pDN0.8, pSB961, pSB981, pSB1006, pSB1025, pSB10313, pSB1047, pSB1083 and pSB1291 using universal M13 forward, universal M13 reverse, SB108.3F2 and SB108.3R2 primers. 6.8kbcontig1 - 6.8kbcontig12 primers were used in sequencing reactions with pDN488L plasmid DNA. ZooA SBD primer 1 was designed from the previously reported *zooA* sequence (Simmonds *et al* (1997)). ZooA SBD primer 1 was used in sequencing reactions with pSB981 plasmid DNA. Sequencing reactions were performed by the Centre for Gene Research (University of Otago, Dunedin, NZ) using an Applied Biosystems (ABI) 373 Version 3.0 DNA sequencer and the manufacturers' procedures and specifications.

Sequence analysis.

DNA sequence analysis was performed using an series 6100/66 Power Macintosh Apple computer. The sequence chromatographs were viewed and trimmed using the SeqEd (ABI) application. DNA sequences were compiled and a contiguous sequence was constructed using the DNAstar Seqman application. Open reading frames and putative amino acid sequences were determined using the DNAstar EditSeq application and visualized using either the DNAstar MapDraw or GeneJockey

(Biosoft, Cambridge, England) applications. DNA and amino acid sequence homology searches were performed using the non-redundant protein and nucleotide databases and the gapped basic local alignment search tool (BLAST) program of the National Centre for Biotechnology Information (NCBI) (NCBI, Bethesda, MD, USA). Sequence alignments and sequence similarity calculations were performed using the DNAstar Megalign application.

Results and Technical Discussion

Transformation of *E. coli* DH5αF' and characterization of transformants.

E. coli DH5αF' were transformed by electro-poration with Bluescript® II SK(+) phagemid vector with a transformation efficiency of approximately 10^6 transformants per μg plasmid DNA. Transformation efficiency for the electro-transformations of pSB1006, pSB1014, pSB1025, pSB10313, pSB1083, and pSB1097 were less than 20 transformants per μg plasmid DNA. All other recombinant Bluescript® II SK(+) phagemid vectors gave transformation efficiencies of between 10^3 - 10^4 transformants per μg plasmid DNA. 2 - 50% of *E. coli* DH5αF' pBluescript® II SK(+) phagemid vector transformants screened on LBA+Ap containing IPTG and X-gal produced white colonies. 5 - 100% of white transformants were initially characterized as containing the predicted recombinant pBluescript® II SK(+) phagemid vector. All pBluescript® II SK(+) phagemid vectors characterized by restriction analysis yielded banding patterns consistent with those predicted by the cloning strategy. The discrepancies observed between *E. coli* DH5αF' transformation efficiency and the number of isolates characterized as possessing plasmids with inserts were considered to be the result of minor variations in miniprep preparations, restriction digestion, gel extraction, ligation, and/or electro-poration.

pBluescript® II SK(+) phagemid vector subclones that involved self-ligation were the simplest to characterize. Although all arose from low efficiency transformations almost 100% of white colonies were shown to carry plasmids with an appropriate insert. In contrast, many of the isolates obtained from higher efficiency transformations were difficult to characterize because of the high background of blue colonies, and the lower proportion (as few as 5%) of white colonies that were

subsequently shown to possess plasmids with an appropriate insert. The high background of blue colonies most likely arose as vectors cleaved with a single restriction enzyme recircularised due to incomplete phosphatase treatment. The high proportion of white colonies that did not harbour inserts was probably related to the use of LBA+Ap containing IPTG and X-gal plates unevenly spread with IPTG or X-gal, or the use of plates not prepared on the day of transformation.

E. coli DH5αF' were transformed by electro-poration with pVA838 with an efficiency of 10^4 - 10^5 transformants per μg plasmid DNA. Electro-competent *E. coli* DH5αF' were transformed with pSB1311 and pSB1847 with an efficiency of less than 10 transformants per μg plasmid DNA. 100% of *E. coli* DH5αF' transformants that grew overnight on LBA+Em500, but not on LBA+Cm and were characterized by restriction analysis of plasmid DNA were shown to contain the predicted recombinant pVA838 vector. *E. coli* DH5αF' were naturally partially resistant to erythromycin and very high concentrations were required to enable selection of pVA838 transformants expressing erythromycin resistance genes. It was noted that colonies that grew rapidly (within 12 - 16 hours) on LBA+250Em transformation plates were far more likely to contain pVA838 or recombinant pVA838 than those that grew after 16 hours. Only pVA838 or recombinant pVA838 transformants were subsequently able to grow on LBA+500Em overnight.

The genetic techniques used in the production of pSB1311 and pSB1847 transformants were essentially the same as those used to produce pBluescript® II SK(+) phagemid vector subclones. Presumably due to the low copy of pVA838, plasmid miniprep yields were only 25% of those obtained from minipreps of pBluescript® II SK(+) phagemid vector subclones. Doubling the amount of culture used to 3 ml increased yields, but increasing the volume of culture beyond 3 ml did not significantly enhance yield. Quantum prep™ uses an adaptation of the standard alkaline lysis miniprep method (Sambrook *et al* (1989)) so there is a limit to the amount of cells that can effectively be lysed without increasing the volume of lysis buffer that is added at the same time. It is most likely that inefficient ligation due to their larger size caused the low transformation efficiencies observed with pSB1311 and pSB1847.

Construction of *E. coli*DH5αF' subclones.

All *E. coli*DH5αF' subclones were constructed without difficulty. pVA838 has two restriction sites within the chloramphenicol resistance determinant that are suitable for shuttle cloning between *E. coli* DH5αF' and *S. gordonii* DL1 ie. *Eco*R I and *Pvu* II. Use of the *Eco*R I site enabled pSB1311 to be constructed without difficulty. In contrast it was more difficult to decide the best strategy to use in constructing pSB1847. Although it was possible to use the *Pvu* II restriction sites flanking the pSB1291 MCS to directly transfer the 4.0 kb insert into pVA838 cleaved with *Pvu* II, this strategy was not favoured for a number of reasons. It has been reported that ligating fragments with two blunt termini, as opposed to one blunt and one overhanging terminus, is less efficient. Also, pSB1311 did not contain the *lac* promoter region and there was uncertainty about the effect that its inclusion into the new construct would have on the expression of *zif*. By using only streptococcal DNA to construct pSB1847 there was little doubt that any observed gene expression was initiated from a streptococcal promoter carried on the 4.0 kb insert and that any phenotypic differences observed between *S. gordonii* DL1 (pSB1311) and (pSB1847) transformants were a consequence of the additional 2.8 kb of DNA carried by pSB1311.

Transformation of *S. gordonii* DL1 and characterization of transformants.

Transformation of *S. gordonii* DL1 with pVA838 gave a transformation efficiency of 10^3 transformants per μg plasmid DNA. Transformation of *S. gordonii* DL1 with pSB1311 or pSB1847 gave an efficiency of less than 10 transformants per μg plasmid DNA. Because of the low efficiency of transformation all transformants suspected of carrying a recombinant pVA838 plasmid were phenotypically characterized. Restriction analysis showed plasmid DNA extracted from transformed *S. gordonii* DL1 to be identical to that obtained from the respective *E. coli* DH5aF' strain.

The low transformation efficiency obtained with pSB1311 and pSB1847, but not with pVA838 transformations of *S. gordonii* DL1 is unlikely to be due to genes carried on the respective inserts as transformants appeared normal in all respects other than their zoocin A resistant zoocin A producer phenotype. pVA838 in *S. gordonii* DL1 was very stable, and pSB1311 and pSB1847 were also able to be

maintained without antibiotic selection. It is more likely that the larger size of pSB1311 and pSB1847 made DNA uptake by competent *S. gordonii* DL1 cells less efficient.

Phenotypic characterization of strains.

The results of the testing of strains for production of and sensitivity to zoocin A by deferred antagonism are given in Table 3. That the inhibitory profile produced by *S. equi* subsp. *zooepidemicus* 4881 was the same as that produced by *S. gordonii* DL1 carrying pSB1311 but not *S. gordonii* DL1 carrying pSB1847 confirming that *zooA* is essential for zoocin A production. A partially purified preparation of zoocin A produced endpoint titres of 2048, 128, 128, 0, 0, 0 and 0 when tested by SSM against standard indicator I2, *S. gordonii* DL1, *S. gordonii* DL1 (pVA838), *S. gordonii* DL1 (pSB1847), *S. gordonii* DL1 (pSB1311), standard indicator I1 and *S. equi* subsp. *zooepidemicus* 4881 respectively.

A summary of the results of the phenotypic testing of *S. gordonii* DL1 transformants is given in Table 4.

Sequence data and sequence analysis.

The subcloning strategy used enabled much of the 6.8 kb *Eco*R I fragment sequence to be established by sequencing from both ends of each subclone from M13 universal forward and reverse primers. Three internal primers were required to complete the single stranded contiguous sequence of the entire 6.8 kb fragment. Fragments carried by pSB1083 and pSB981 were too large to be sequenced completely with the M13 universal primers, consequently SB1083R2 and SB1083F2 primers were designed to enable sequencing of the remaining undetermined region within the 2.3 kb pSB1083 insert. SBD primer 1 was used to complete the sequencing of pSB981. To obtain a double stranded contiguous sequence the 6.8kbcontig1 - 12 primers were designed and used in sequencing reactions with pDN488L.

The nucleotide sequence of the 6.8 kb *Eco*R I fragment is given in Figure 3 and the identified open reading frames (ORF) are given in Figure 2. Sequence analysis indicated the prescence of an ORF encoding a 411 amino acid protein (including the "stop" residue) which we have called *zif*. (zoocin A immunity factor). That *zif* is

essential for zoocin A immunity is supported by the observation that zoocin A inhibited *S. gordonii* DL1 and *S. gordonii* DL1 pVA838, but not *S. gordonii* DL1 carrying pSB1311 or pSB1847. *zif* is located on the 4.0 kb *Eco*R I - *Pst* I fragment of pDN488L that is common to both pSB1311 and pSB1847.

Three further ORFs were identified (Figure 2). ORF 1 encodes a 142 amino acid sequence with homology to the 5' region of *rgg* which regulates expression of glucosyltransferase in *S. gordonii* CH1. ORF 2 encodes a 244 amino acid sequence with homology to insertion sequence IS200 found in a range of bacteria including *Clostridium perfringens, E. coli,* and *Yersinia pestis*. However, ORF 2 is most closely related to an IS200 sequence identified in *S. pneumoniae*. ORF 3 encodes a 394 amino acid sequence with homology to a transposase/insertion sequence also identified in *S. pneumoniae*.

Table 1. Bacterial strains and plasmids used in this study.

Species, strain, and (plasmid)	Size (kb) of		Selective antibiotic[c]	Strain and plasmid references
	Plasmid	Insert		
E. coli				
XL1-blue (pDN0.8)[a]	3.5	0.6	Ap100	Simmonds *et al* (1997)
XL1-blue (pDN2.2)[a]	5.1	2.2	Ap100	Simmonds *et al* (1997)
XL1-blue (pDN488L)[a]	9.7	6.8	Ap100	Simmonds *et al* (1997)
DH5aF' (pSK®II(+))[a]	2.9	No insert	Ap100	Woodcock *et al* (1989); Raleigh *et al* (1989); Alting-Mees and Short (1989); Short *et al* (1988)
DH5aF' (pSB961)[a]	3.6	0.7	Ap100	herein
DH5aF' (pSB981)[a]	4.4	1.5	Ap100	herein
DH5aF' (pSB1006)[a]	6.6	3.7	Ap100	herein
DH5aF' (pSB1025)[a]	6.3	3.4	Ap100	herein
DH5aF' (pSB1014)[a]	6.0	3.1	Ap100	herein
DH5aF' (pSB10313)[a]	3.7	0.8	Ap100	herein
DH5aF' (pSB1047)[a]	3.1	0.2	Ap100	herein
DH5aF' (pSB1083)[a]	5.2	2.3	Ap100	herein
DH5aF' (pSB1097)[a]	3.2	0.3	Ap100	herein
DH5aF' (pSB1291)[a]	6.9	4.0	Ap100	herein Macrina *et al* (1982)
DH5aF' (pVA838)[b]	9.2	No insert	Cm25, Em500	herein
DH5aF' (pSB1311)[b]	16.0	6.8	Cm25, Em500	herein
DH5aF' (pSB1847)[b]	13.2	4.0	Cm25, Em500	herein
S. gordonii				
DL1 (pVA838)[b]	9.2	No insert	Em10	Macrina *et al* (1982)
DL1 (pSB1311)[b]	16.0	6.8	Em10	herein
DL1 (pSB1847)[b]	13.2	4	Em10	herein

a Parent vector, pBluescript® II SK(+) phagemid vector (Stratagene).

b Parent vector, pVA838 (kindly donated by Dr H. Jenkinson, Dept. of Oral Biology, University of Otago, Dunedin, NZ).

c Antibiotic abbreviations; Ap100, 100 mg/ml ampicillin; Cm25, 25 mg/ml chloramphenicol; Em500, 500 mg/ml erythromycin and Em10, 10 mg/ml erythromycin.

Table 2. Primers used in this study.

Primer		
Designation	sequence[a]	position & orientation
Universal M13 reverse	GGAAACAGCTATGACCATG	806 (+)[b]
Universal M13 forward	GTAAAACGACGGCCAGT	579 (-)[b]
SB108.3R2	TGAGTGAAGCAACTG	1214 (+)[c]
SB108.3F2	TTATGCTCCAGCACT	2680 (-)[c]
ZooA SBD primer 1	GGGTTGATAATGG	4547 (+)[c]
6.8kbcontig1	AGTCTGTAGGTTCGTATTCT	1375 (-)[c]
6.8kbcontig2	TGTGGCTTCATTAGGTCCAA	1754 (+)[c]
6.8kbcontig3	AGTACTGTTGGACCTAATGA	1780 (-)[c]
6.8kbcontig4	TGCGGGTGCGCGACGAAGGT	2212 (-)[c]
6.8kbcontig5	TTGGGTATAACCTTCGTCGC	2184 (+)[c]
6.8kbcontig6	TTCCCAGTAATACCTAACAT	2592 (+)[c]
6.8kbcontig7	TCATAATACTCAAGTCCTTT	3024 (+)[c]
6.8kbcontig8	AATATCAAGTTCTAATACAT	3375 (+)[c]
6.8kbcontig9	TCAATCTTGTCTCTGTCCTT	5050 (+)[c]
6.8kbcontig10	CGTCTTTTGAGCTACTCTGA	5231 (-)[c]
6.8kbcontig11	GGCGAATCAAAGTCTTGTAG	5910 (+)[c]
6.8kbcontig12	TTCTCGATTGCGCAGGCTAC	5945 (-)[c]

a Primer sequence is presented 5' to 3'.

b Primer position is given as the first nucleotide of the primer relative to the sequence of the pBluescript® II SK (+) phagemid vector as previously described (Short *et al*, 1988; Alting-Mees *et al*, 1989).

c Primer position is given as the first nucleotide of the primer relative to the sequence of the 6.8 kb fragment of pDN488L as designated in Figure 3.

Table 3. Production and sensitivity to BLIS of strains tested by deferred antagonism.

Indicator strains	Producer strain				
	4881[a]	DL1[b]	pVA838[c]	pSB1311[d]	pSB1847[e]
I1	-	-	-	-	-
I2	+	-	-	+	-
I3	-	-	-	-	-
I4	-	-	-	-	-
I5	+	-	-	+	-
I6	-	-	-	-	-
I7	+	-	-	+	-
I8	+	-	-	+	-
I9	+	-	-	+	-
4881	-	-	-	-	-
DL1	+	-	-	+	-
pVA838	+	-	-	+	-
pSB1311	-	-	-	-	-
pSB1847	-	-	-	-	-

a *S. equi* subsp. *zooepidemicus* 4881.
b *S. gordonii* DL1.
c *S. gordonii* DL1 (pVA838).
d *S. gordonii* DL1 (pSB1311).
e *S. gordonii* DL1 (pSB1847).

Table 4. Phenotypic characterization of *S. gordonii* DL1 clones.

		Phenotype		
Strain and plasmid	Genotype[a]	Zoocin A production	Zoocin A immunity	Em resistance[b]
S. gordonii				
DL1	*zooA* - *zif* - Em^S	-	-	-
DL1 (pVA838)	*zooA* - *zif* - Em^R	-	-	+
DL1 (pSB1311)	*zooA* + *zif* + Em^R	+	+	+
DL1 (pSB1847)	*zooA* - *zif* + Em^R	-	+	+
S. equi subsp.*zooepidemicus*				
4881	*zooA* + *zif* + Em^S	+	+	-

a *zooA* +/- denotes the prescence or absence of the gene encoding zoocin A, *zif* +/- denotes the prescence or absence of the gene encoding zoocin A immunity, Em^R denotes the presence of the erythromycin resistance gene located on pVA838 and Em^S indicates no erythromycin resistance gene.

b Denotes sensitivity or resistance to 10 μg/ml erythromycin.

The foregoing examples are illustrations of the invention. The invention may be carried out with numerous variations and modifications as will be apparent to those skilled in the art. For example, the native *zif* gene need not be used in the transformation. Deletions, insertions and substitutions relative in the *zif* gene may be used provided that the *zif*-type activity is retained. Similarly the gene may be incorporated into species other than used in Example 1. Likewise there are many variations in the way in which the invention can be used in pharmaceuticals and food products.

REFERENCES

Alting-Mees, M.A. and J.M. Short. 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. 17: 9494.

Altschul, S.F. *et al.* 1990. J. Mol. Biol., 215:403-410.

Bronze, M.S. and J.B. Dale. 1996. The reemergence of serious group A streptococcal infections and acute rheumatic fever. Am. J. Med. Sci. 311: 41-54.

Dower, W.J. 1988. Transformation of *E. coli* to extremely high efficiency by electroporation. Mol. Biol. Rep. 6: 3-4.

Federal Register. 1988. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed. Regist. 54: 11247-11251.

Francis, A.J., G.R. Nimmo, A. Efstratiou, V. Galanis and N. Nuttall. 1993. Investigation of milk-borne *Streptococcus zooepidemicus* infection associated with glomerulonephritis in Australia. J. Infect. 27: 317-323.

Jack, R.W. 1991. Production, purification and characterisation of the streptococcal lantibiotic streptococcin A-FF22. A Thesis. University of Otago, Dunedin, New†Zealand.

Jones C.L., J.A. Ritchie, P.D. Marsh and F.J.G. van der Ouderaa. 1988. The effect of long-term use of a dentifice containing zinc citrate and a non-ionic agent on the oral flora. J. Dent. Res. 67: 46-50.

Loesche, W.J. 1976. Chemotherapy of dental plaque infections. Oral Sci. Rev. 9: 65-107.

Loesche, W.J., S.A. Eklund, D.F. Mehlisch and B. Burt. 1989. Possible effects of medically administered antibiotics on the mutans streptococci, implications for reduction in decay. Oral Microbiol. Immunol. 4: 77-81.

Macrina, F.L., J.A. Tobian, K.R. Jones, R.P. Evans and D.B. Clewell. 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene. 19: 345-353.

Marsh, P.D. 1991. Dentifrices containing new agents for the control of plaque and gingivitis: microbiological aspects. Clin. Periodontol. 18: 462-467.

Pearson, W.R. *et al.*, 1988. Proc. Natl. Acad. Sci., 85:2444-2448.

Raleigh, E.A., K. Lech and R. Brent. 1989. Selected topics from classical bacterial genetics. In. Current protocols in molecular biology. eds. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. Publishing Associates and Wiley Interscience, New York. Unit 1.4.

Rodriguez, J.M. and H.M. Dodd. 1996. Genetic determinants for the biosynthesis of nisin, a bacteriocin produced by *Lactococcus lactis*. Microbiologia. 12: 61-74.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, USA.

Schofield, C.R. and J.R. Tagg. 1983. Bacteriocin-like activity of group B and group C streptococci of human and of animal origin. J. Hyg. 90: 7-18.

Short, J.M., J.M. Fernandez, J.A. Sorge and W.D. Huse. 1988. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. Nucleic Acids Res. 16: 7583-7600.

Simmonds, R.S., J. Naidoo, C.L. Jones and J.R. Tagg. 1995. The streptococcal bacteriocin-like inhibitory substance, zoocin A, reduces the proportion of *Streptococcus mutans* in an artificial plaque. Microb. Ecol. Health Dis. 8: 281-292.

Simmonds, R.S., Simpson, W.J. and Tagg J.R. 1997. Cloning and sequence analysis of *zooA*, a *Streptococcus zooepidemicus* gene encoding a bacteriocin-like inhibitory

substance having a domain structure similar to that of lysostaphin. Gene. 189: 255-261.

Simmonds R.S., L. Pearson, R.C. Kennedy and J.R. Tagg. 1996. Mode of action of a lysostaphin-like bacteriolytic agent produced by *Streptococcus zooepidemicus* 4881. Appl. Environ. Microbiol. 62: 4536-4541.

Tagg, J.R. and L.V. Bannister. 1979. "Fingerprinting" b-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. J. Med. Microbiol. 12: 397-411.

Thumm, G. and F. Gotz. 1997. Studies on prolysostaphin processing and characterization of the lysostaphin immunity factor (Lif) of *Staphylococcus simulans* biovar *staphylolyticus*. Mol. Microbiol. 23: 1251-1265.

Vriesema, A.J.M., S.A.J. Zaat and J. Dankert. 1996. A simple procedure for isolation of cloning vectors and endogenous plasmids from viridans group streptococci and *Staphylococcus aureus*. Appl. Environ. Microbiol. 62: 3527-3529.

Woodcock, D.M., P.J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S.S. Smith, M.Z. Michael and M.W. Graham. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res. 17: 3469-3478.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Otago
New Zealand Pastoral Agriculture Research Institute Limited

(ii) TITLE OF INVENTION: Zoocin A Immunity Factor

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESS: Russell McVeagh West-Walker
(B) STREET: The Todd Building, Cnr Brandon Street and Lambton Quay
(C) CITY: Wellington
(D) COUNTRY: New Zealand

(v) COMPUTER READABLE FORM
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: Windows 95

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: NZ 329227
(B) FILING DATE: 21 November 1997

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bennett, Michael Roy
(B) REFERENCE/DOCKET NUMBER: 23804 MRB

(viii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 64 4 499 9058
(B) TELEFAX: 64 4 499 9306

(2) INFORMATION FOR SEQ ID NO. 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 410 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

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                Met Lys Phe Gln Glu Ile Asp Ala Leu
                                    5
Thr Phe Glu Lys Phe Ala Asn Thr Gln Lys Arg Arg Ser
10                  15                  20
Phe Glu Gln Thr Ile Glu Met Gly Asn Leu Arg Lys Ser
        25                  30                  35
Arg Asn Phe Asp Val Lys Tyr Phe Ala Leu Phe His Leu
                40                  45
Glu Glu Ile Lys Val Val Ala Leu Thr Tyr Thr Gln Lys
    50                  55                  60
Ile Phe Gly Gly Leu Asn Met Gly Ile Tyr Tyr Gly Pro
                65                  70
Ile Phe Ser Glu Glu Arg Tyr Leu Ala His Phe Leu Ile
75                  80                  85
Glu Leu Lys Lys Tyr Thr Lys Lys Asn Asn Val Leu Glu
        90                  95                  100
Leu Asp Ile Phe Pro Tyr Asp Asp Tyr Gln Tyr Tyr Asp
                105                 110
Asp Glu Gly Arg Leu Ile Gln Asp Gly Asn Ile Glu Leu
    115                 120                 125
Arg Asp Ile Phe Glu Lys Ala Gly Phe Thr Tyr Gln Gly
            130                 135
Asp Glu Val Gly Phe Asn Ser Glu Gln Val Thr Trp His
140                 145                 150
Tyr Val Lys Asp Leu Thr Asn Leu Thr Ser Glu Asn Leu
        155                 160                 165
Leu Asn Ser Phe Ser Lys Lys Gly Arg Pro Leu Val Lys
                170                 175
Lys Ser Asn Thr Phe Gly Ile Lys Val Arg Lys Leu Asn
    180                 185                 190
Lys Asp Glu Leu Gln Ile Phe Ala Asn Ile Thr Asn Asp
            195                 200
Thr Ala Thr Arg Arg Gly Tyr Asn Asp Lys Gly Leu Glu
205                 210                 215
Tyr Tyr Glu Lys Phe Phe Asp Ala Phe Lys Asp Lys Ser
        220                 225                 230
Glu Phe Thr Ile Ala Thr Leu Asn Phe Arg Glu Tyr Leu
                235                 240
Gly Asn Ile Leu Asp Gly Arg His Arg Leu Glu Asn Lys
    245                 250                 255
Ile Ser Ile Leu Gly Thr Arg Leu Asp Lys Asn Pro Asn
            260                 265
Ser Glu Lys Ile Lys Asn Gln Leu Arg Glu Leu Asn Ser
270                 275                 280
Gln Arg Glu Thr Phe Leu Ile Arg Glu Glu Glu Ala Lys
        285                 290                 295
Ser Phe Val Lys Lys Tyr Gly Asp Glu Asp Val Val Leu
                300                 305
Ala Gly Ser Leu Phe Val Tyr Thr Gln Gln Glu Leu Val
    310                 315                 320
```

```
Tyr Leu Tyr Ser Gly Ser Tyr Val Glu Phe Asn Lys Phe
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Tyr Ala Pro Ala Leu Leu Gln Glu Tyr Ala Met Leu Asn
335                 340                 345
Ala Leu Lys Lys Gly Ile Lys Phe Tyr Asn Met Leu Gly
        350                 355                 360
Ile Thr Gly Lys Phe Asp Asn Ser Asp Gly Val Leu Cys
                365                 370
Phe Lys Gln Asn Phe Lys Gly Tyr Ile Val Arg Lys Phe
    375                 380                 385
Ser Asn Phe Ile Tyr Tyr Pro Asn Pro Arg Lys Leu Lys
            390                 395
Val Ile Gln Leu Ile Lys Ser Ile Leu Arg Arg
400                 405                 410
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(2) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1230 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

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TCAGAAAAGA CGTTCTTTTG AGCAAACCAT TGAAATGGGA AATTTAAGAA     100

AGAGTCGAAA TTTTGATGTT AAATATTTTG CTCTTTTTCA TTTGGAGGAA     150

ATAAAGGTTG TCGCACTTAC ATATACCCAA AAAATATTTG GTGGCTTGAA     200

TATGGGTATT TATTATGGAC CTATTTTTAG TGAAGAAAGA TATCTTGCAC     250

ATTTTTTGAT TGAATTAAAA AAATATACGA AAAAAAATAA TGTATTAGAA     300

CTTGATATTT TTCCATATGA TGATTATCAA TATTATGATG ATGAAGGTAG     350

GTTAATTCAA GATGGTAATA TTGAATTAAG AGATATTTTT GAAAAAGCTG     400

GTTTTACATA TCAGGGGGAT GAAGTTGGTT TTAATAGTGA GCAAGTAACT     450

TGGCATTATG TTAAAGATTT AACTAATCTT ACATCAGAAA ATCTACTAAA     500

TTCATTTTCA AAAAAAGGAC GTCCGTTAGT AAAAAAATCT AATACTTTTG     550
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GAATAAAAGT TAGAAAGCTT AATAAAGATG AACTTCAAAT ATTTGCAAAT 600

ATAACAAATG ATACAGCCAC TCGTCGAGGT TATAATGACA AAGGACTTGA 650

GTATTATGAA AAATTTTTCG ATGCATTTAA AGATAAGTCA GAATTTACTA 700

TTGCAACTTT GAATTTCCGT GAGTATTTAG GCAATATATT GGATGGTCGA 750

CATAGGCTTG AGAATAAAAT TTCAATTTTA GGCACTAGGT TAGATAAAAA 800

TCCAAACTCT GAAAAAATAA AAAATCAACT TAGAGAGTTA AATAGTCAAC 850

GAGAAACATT TTTAATTAGA GAAGAAGAAG CGAAATCTTT TGTTAAGAAG 900

TATGGTGATG AGGATGTCGT TCTTGCGGGA AGCCTTTTTG TATATACTCA 950

GCAAGAATTA GTATATCTTT ATTCAGGCTC ATATGTGGAG TTTAACAAGT 1000

TTTATGCTCC AGCACTTTTA CAAGAATATG CTATGTTAAA TGCATTAAAA 1050

AAAGGAATAA AATTTTATAA TATGTTAGGT ATTACTGGGA AATTTGATAA 1100

TTCAGATGGT GTTCTATGTT TTAAACAGAA CTTTAAGGGG TATATAGTTC 1150

GTAAGTTTTC AAATTTTATT TACTACCCAA ACCCTAGAAA ATTAAAAGTT 1200

ATACAACTAA TTAAAAGCAT TTTGAGAAGG 1230

(2) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

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                                                      5
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20                  25                  30
Gly Asn Ile Thr Thr Gly Phe Asn Gly Tyr Pro Gly His
          35                  40                  45
Val Gly Val Asp Tyr Ala Val Pro Val Gly Thr Pro Val
                    50                  55
Arg Ala Val Ala Asn Gly Thr Val Lys Phe Ala Gly Asn
      60                  65                  70
Gly Ala Asn His Pro Trp Met Leu Trp Met Ala Gly Asn
                 75                  80
Cys Val Leu Ile Gln His Ala Asp Gly Met His Thr Gly
85                  90                  95
Tyr Ala His Leu Ser Lys Ile Ser Val Ser Thr Asp Ser
          100                 105                 110
Thr Val Lys Gln Gly Gln Ile Ile Gly Tyr Thr Gly Ala
                    115                 120
Thr Gly Gln Val Thr Gly Pro His Leu His Phe Glu Met
     125                 130                 135
Leu Pro Ala Asn Pro Asn Trp Gln Asn Gly Phe Ser Gly
                 140                 145
Arg Ile Asp Pro Thr Gly Tyr Ile Ala Asn Ala Pro Val
150                 155                 160
Phe Asn Gly Thr Thr Pro Thr Glu Pro Thr Thr Pro Thr
          165                 170                 175
Thr Asn Leu Lys Ile Tyr Lys Val Asp Asp Leu Gln Lys
                    180                 185
Ile Asn Gly Ile Trp Gln Val Arg Asn Asn Ile Leu Val
     190                 195                 200
Pro Thr Asp Phe Thr Trp Val Asp Asn Gly Ile Ala Ala
                 205                 210
Asp Asp Val Ile Glu Val Thr Ser Asn Gly Thr Arg Thr
215                 220                 225
Ser Asp Gln Val Leu Gln Lys Gly Gly Tyr Phe Val Ile
          230                 235                 240
Asn Pro Asn Asn Val Lys Ser Val Gly Thr Pro Met Lys
                    245                 250
Gly Ser Gly Gly Leu Ser Trp Ala Gln Val Asn Phe Thr
     255                 260                 265
Thr Gly Gly Asn Val Trp Leu Asn Thr Thr Ser Lys Asp
                 270                 275
Asn Leu Leu Tyr Gly Lys
280                 285
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(2) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 855 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

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GGATACCCTG GTCATGTTGG AGTCGATTAT GCAGTACCCG TTGGAACTCC     170

GGTTAGAGCA GTTGCAAATG GTACAGTCAA ATTTGCAGGT AATGGGGCTA     220

ATCACCCATG GATGCTTTGG ATGGCTGGAA ACTGTGTTCT AATTCAACAT     270

GCTGACGGGA TGCATACTGG ATATGCACAC TTATCAAAAA TTTCAGTTAG     320

CACAGATAGT ACAGTTAAAC AAGGACAAAT CATAGGTTAT ACTGGTGCCA     370

CCGGCCAAGT TACCGGTCCA CATTTGCATT TTGAAATGTT GCCAGCAAAT     420

CCTAACTGGC AAAATGGTTT TTCTGGAAGA ATAGATCCAA CCGGATACAT     470

CGCTAATGCC CCTGTATTTA ATGGAACAAC ACCTACAGAA CCTACTACTC     520

CTACAACAAA TTTAAAAATC TATAAAGTTG ATGATTTACA AAAAATTAAT     570

GGTATTTGGC AAGTAAGAAA TAACATACTT GTACCAACTG ATTTCACATG     620

GGTTGATAAT GGAATTGCAG CAGATGATGT AATTGAAGTA ACTAGCAATG     670

GAACAAGAAC CTCTGACCAA GTTCTTCAAA AAGGTGGTTA TTTTGTCATC     720

AATCCTAATA ATGTTAAAAG TGTTGGAACT CCGATGAAAG GTAGTGGTGG     770

TCTATCTTGG GCTCAAGTAA ACTTTACAAC AGGTGGAAAT GTCTGGTTAA     820

ATACTACTAG CAAAGACAAC TTACTTTACG GAAAA                     855
```

CLAIMS

1. A protein which comprises the amino acid sequence of SEQ ID NO. 1 and which is capable of protecting a host cell expressing it against zoocin A activity, or a functionally equivalent variant thereof.

2. A protein as claimed in claim 1 which has the amino acid sequence of SEQ ID NO. 1.

3. A DNA molecule which encodes a protein as claimed in claim 1.

4. A DNA molecule which comprises SEQ ID NO. 2, or a functionally equivalent variant thereof.

5. A vector which includes a DNA molecule as claimed in claim 3 or claim 4.

6. A vector as claimed in claim 5 which further includes DNA encoding a protein having zoocin A activity.

7. A vector as claimed in claim 6 wherein said protein having zoocin A activity has or includes the amino acid sequence of SEQ ID NO. 3, or a functionally equivalent variant thereof.

8. A vector as claimed in claim 6 wherein said DNA encoding said protein has or includes the nucleotide sequence of SEQ ID NO. 4, or a functionally equivalent variant thereof.

9. A method of protecting an organism susceptible to the bacteriolytic activity of zoocin A against such activity which comprises the step of introducing into said organism a DNA molecule according to claim 3 or claim 4.

10. A method as claimed in claim 9 wherein said DNA molecule is introduced into said organism in the form of a vector as claimed in claim 5.

11. An organism which has been rendered resistant to zoocin A activity by a method as claimed in claim 9 or claim 10.

12. A method of genetically modifying a non-pathogenic organism to express a protein having zoocin A activity without said organism being itself at risk

from said activity which comprises the step of introducing a DNA molecule encoding said protein into an organism as claimed in claim 11.

13. A method of genetically modifying a non-pathogenic organism to express a protein having zoocin A activity without said organism being itself at risk from said activity which comprises the step of introducing into said organism a DNA molecule encoding said protein together with a DNA molecule according to claim 3 or claim 4.

14. A method as claimed in claim 13 wherein said DNA molecules are introduced into said organism in the form of a vector as claimed in any one of claims 6 to 8.

15. A non-pathogenic organism which has been genetically modified in accordance with a method as claimed in any one of claims 12 to 14.

16. A non-pathogenic organism which is resistant against zoocin A activity and wherein said resistance is due to the presence in said organism of a DNA molecule as claimed in claim 3 or claim 4.

17. A non-pathogenic organism which expresses a protein having zoocin A activity but which is itself resistant to said activity, wherein said resistance is due to the presence in said organism of a DNA molecule as claimed in claim 3 or claim 4.

18. A non-pathogenic organism as claimed in claim 17 which is a food grade organism.

19. A non-pathogenic organism as claimed in claim 18 which is a food grade *Streptococcus*.

20. A non-pathogenic organism as claimed in claim 19 wherein the food grade *Streptococcus* is *S. gordonii*.

21. An antibacterial composition which comprises a non-pathogenic organism according to any one of claims 15 and 17 to 20.

22. An antibacterial composition as claimed in claim 21 which is suitable for human ingestion.

23. An antibacterial composition as claimed in claim 21 which is suitable for ingestion by a non-human animal.

24. An antibacterial composition as claimed in claim 22 or claim 23 which is, or is part of, a foodstuff.

25. An antibacterial composition as claimed in claim 22 which is, or is part of, a nutriceutical.

26. An antibacterial composition as claimed in claim 24 or claim 25 which is or contains a dairy product.

27. An antibacterial composition as claimed in claim 22 which is, or is part of, a confectionery.

28. An antibacterial composition as claimed in claim 27 which is a wine gum or chewing gum.

29. A method of preventing or inhibiting the growth of undesirable organisms susceptible to zoocin A which comprises the step of contacting said undesirable organisms or the environment thereof with a composition as claimed in claim 21.

30. A method as claimed in claim 29 wherein said composition is administered to the oral cavity of a patient to prevent or inhibit the growth of *S. mutans, S. sobrinus* and/or *S. pyogenes.*

31. A method of treating or preventing *Streptococcal* sore throat or dental caries in a susceptible patient which comprises the step of orally administering to said patient a composition as claimed in claim 22.

Figure 1. Map of pBluescript® II SK(+) phagemid vector and pVA838.

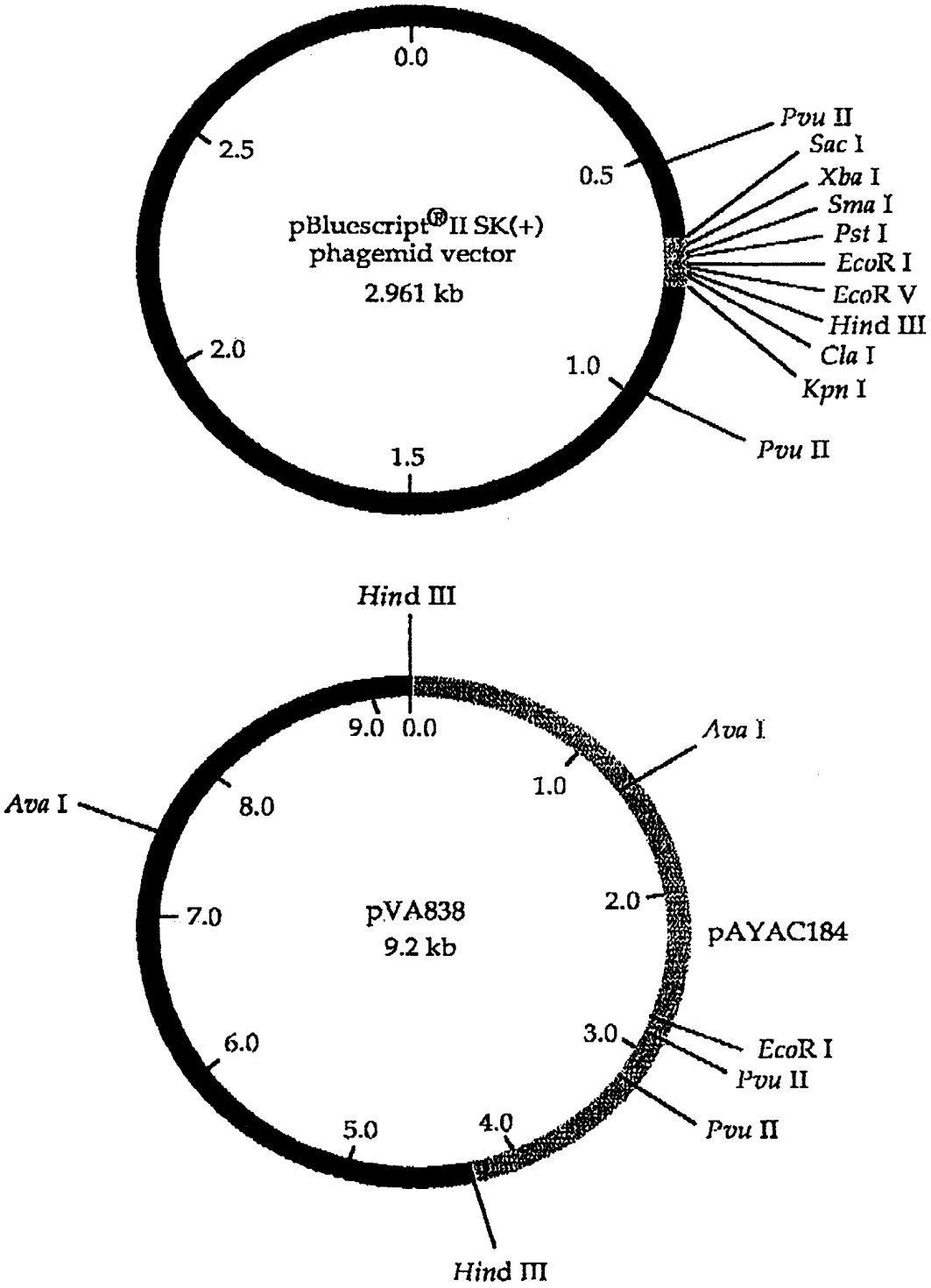
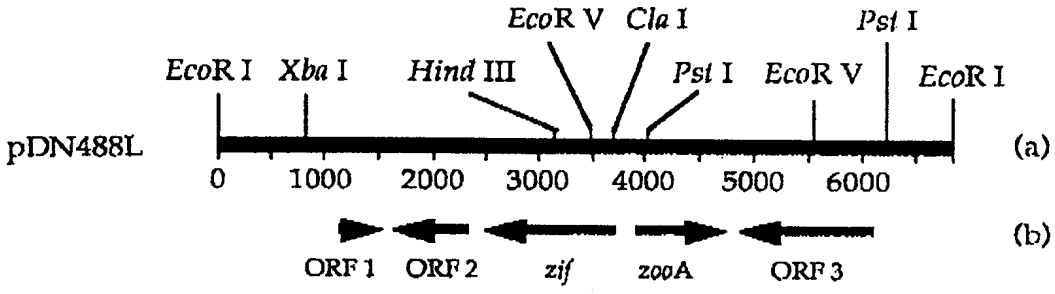
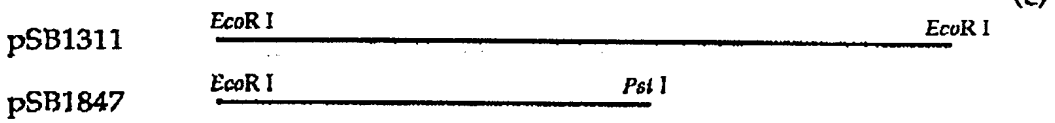


Figure 2. Restriction map of pDN488L showing ORFs and subclones.

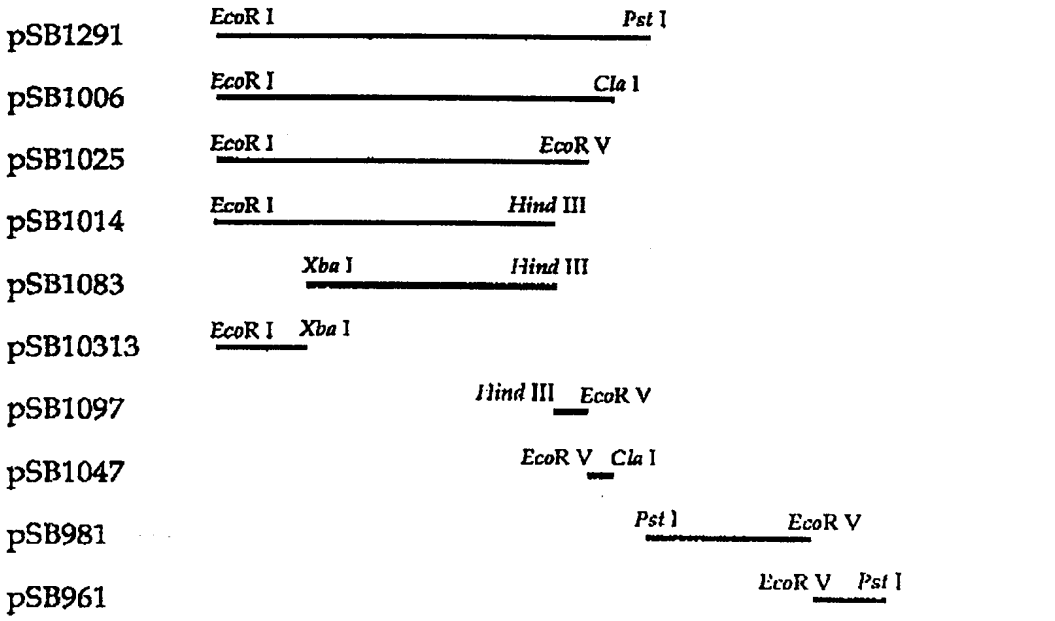


pVA838 subclones.

(c)



pBluescript® II SK(+) subclones.



Previously described pBluescript® II SK(+) subclones.

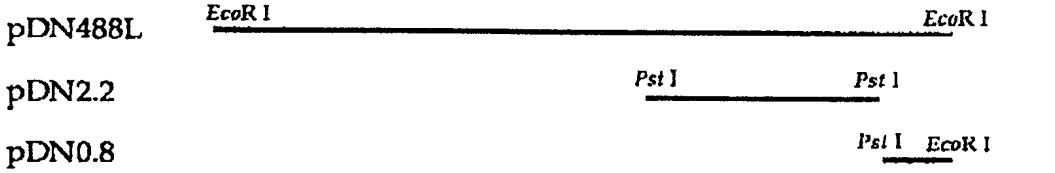


Figure 3. **DNA sequence of 6.8 kb EcoR I fragment showing *zooA* and *zif*.**

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TGAATCACCGGTGCCAATACAGCTTTCCTTACCTCCTTAGCTCAGTTGGT  198
AGAGCAGTAGACTCTTAATCTATGGGTCACAGGTTCGAGCCCTGTAGGGG  248
GTATCATACTATACATAAAAAAGCCTTTAAATAAAGGCTTTTTTGCTTGT  298
CTAAAGAGGATTTGTTCCACCATTTGTCCCCGAAACAATTTTTTATGATA  348
TGATGTTAAATAAAGAATTTATTACTTTTTAAAAAGAGAGCTAACACATG  398
ACAATAATATAATTAACACAAGGTGATACAATCGGAGTATTTTCACCTCC  448
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TATGCTAGGAGAAAAAAAGAGACTTTAAGTGGATGGAGCACCTAGAGAGA  748
CTCGAAAATAATTGGCTTTATAGCCGTATGGCCGAGGAAGAAGTATTACT  798
TCAAAAAATTCTAGATTTTGCAATATTTCAAAATAAATTAGATAAAAACA  848
CTCTAACAACTAAAGAGCTTAATAATGAACTTAATCTTGAAGACAACAAC  898
ACTATGTCCAAAGAAGATAAAATTTCACTCATCATATTGAAATATGAAAA  948
AGAAATTCAAGAAAAAACTCAAAAACTTATTGATTTATATAAAGATCAAT  998
CAAGCAATGAATTAGATACAATAAGATTTGAATCGACTAATATATAATTC 1048
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AAAGGAACTAGATAAAATTAAAATTGATGGTCGAGAACAATATCATAGAC 1498
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GGCTATAAGCCCACATCACCAGCCAGCGCCTAAAGACGCTGGCTTTCACT 1598
TTGTTCAAGCCTCACCGCTTTTGACTCGTCACCAGCCTCTTAAAGAGGCG 1648
TTCGTACTACTTACCATTATCCCTAAAGGGATCTTCATACTCTTTTACAC 1698
TCAATTTATCAAGTGCTATATCATGTTTTTCCTGTTCTTGGATATATTTC 1748
TTAATTGTGGCTTCATTAGGTCCAACAGTACTCACATAATAGCCCTCTGC 1798
CCAAAAATGGCGATTGCCAAATTTGTATTTGAGATTAGGGCGTTTGTCAA 1848
ACATCATCAAAGCGCTCTTTCCTTTCAAATATCCCATGAAACTTGACACA 1898
CTTAATCTCGGAGGAACGCTGACTAACATGTGAACATGGTCTGGCATCAG 1948
ATGACCTTCGATAATTTCAACACCTTTATAACGACACAAGCGTCGGAATA 1998
TTTCTCCCAAACTACTTCGATATTGATTAGAGATGCTTTTTCGTCTATAC 2048
TTAGGTGTAAAGACAATATGGTACTTGCACAACCACTTTGTATGTGATAA 2098
ACTATGTGCCTTTTGTGCCATACTTTTCTCCTTTCACTATACAATAGGCT 2148
TGAACACCTTTATTGTATCGCGTTTGGAGTTTTTTTGGGTATAACCTTCG 2198
TCGCGCACCCGCATAGCGGGTGGTTTATTTGTCTCGCACCTTACGGAGCG 2248
TGACGGACTTAAAGTCACATAATTAAGATAATTTCCTTTTATATATTTCA 2298
GAAAAATATAAAAGGAAATTGATCCTACTCTTGAACTAATTAAGTTAAAT 2348
AATCCCATTAAAAAAGGTATATGCTGTTCTGATAACATAGAAGTATTTAT 2398
GCCTTATTTTTTAATTTTAATAGACATATGAGCTTATAGTTAAATCCCAT 2448

     CTA  CCT TCT CAA AAT GCT TTT AAT TAG TTG TAT AAC  2484
411  Stop Arg Arg Leu Ile Ser Lys Ile Leu Gln Ile Val
```

FIG 3 (Cont'd)


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    TTT TAA TTT TCT AGG GTT TGG GTA GTA AAT AAA ATT TGA   2523
399 Lys Leu Lys Arg Pro Asn Pro Tyr Tyr Ile Phe Asn Ser

    AAA CTT ACG AAC TAT ATA CCC CTT AAA GTT CTG TTT AAA   2562
386 Phe Lys Arg Val Ile Tyr Gly Lys Phe Asn Gln Lys Phe

    ACA TAG AAC ACC ATC TGA ATT ATC AAA TTT CCC AGT AAT   2601
373 Cys Leu Val Gly Asp Ser Asn Asp Phe Lys Gly Thr Ile

    ACC TAA CAT ATT ATA AAA TTT TAT TCC TTT TTT TAA TGC   2640
360 Gly Leu Met Asn Tyr Phe Lys Ile Gly Lys Lys Leu Ala

    ATT TAA CAT AGC ATA TTC TTG TAA AAG TGC TGG AGC ATA   2679
347 Asn Leu Met Ala Tyr Glu Gln Leu Leu Ala Pro Ala Tyr

    AAA CTT GTT AAA CTC CAC ATA TGA GCC TGA ATA AAG ATA   2718
334 Phe Lys Asn Phe Glu Val Tyr Ser Gly Ser Tyr Leu Tyr

    TAC TAA TTC TTG CTG AGT ATA TAC AAA AAG GCT TCC CGC   2757
321 Val Leu Glu Gln Gln Thr Tyr Val Phe Leu Ser Gly Ala

    AAG AAC GAC ATC CTC ATC ACC ATA CTT CTT AAC AAA AGA   2796
308 Leu Val Val Asp Glu Asp Gly Tyr Lys Lys Val Phe Ser

    TTT CGC TTC TTC TTC TCT AAT TAA AAA TGT TTC TCG TTG   2835
295 Lys Ala Glu Glu Glu Arg Ile Leu Phe Thr Glu Arg Gln

    ACT ATT TAA CTC TCT AAG TTG ATT TTT TAT TTT TTC AGA   2874
282 Ser Asn Leu Glu Arg Leu Gln Asn Lys Ile Lys Glu Ser

    GTT TGG ATT TTT ATC TAA CCT AGT GCC TAA AAT TGA AAT   2913
269 Asn Pro Asn Lys Asp Leu Arg Thr Gly Leu Ile Ser Ile

    TTT ATT CTC AAG CCT ATG TCG ACC ATC CAA TAT ATT GCC   2952
256 Lys Asn Glu Leu Arg His Arg Gly Asp Leu Ile Asn Gly

    TAA ATA CTC ACG GAA ATT CAA AGT TGC AAT AGT AAA TTC   2991
243 Leu Tyr Glu Arg Phe Asn Leu Thr Ala Ile Thr Phe Glu

    TGA CTT ATC TTT AAA TGC ATC GAA AAA TTT TTC ATA ATA   3030
230 Ser Lys Asp Lys Phe Ala Asp Phe Phe Lys Glu Tyr Tyr

    CTC AAG TCC TTT GTC ATT ATA ACC TCG ACG AGT GGC TGT   3069
217 Glu Leu Gly Lys Asp Asn Tyr Gly Arg Arg Thr Ala Thr

    ATC ATT TGT TAT ATT TGC AAA TAT TTG AAG TTC ATC TTT   3108
204 Asp Asn Thr Ile Asn Ala Phe Ile Gln Leu Glu Asp Lys

    ATT AAG CTT TCT AAC TTT TAT TCC AAA AGT ATT AGA TTT   3147
191 Asn Leu Lys Arg Val Lys Ile Gly Phe Thr Asn Ser Lys

    TTT TAC TAA CGG ACG TCC TTT TTT TGA AAA TGA ATT TAG   3186
178 Lys Val Leu Pro Arg Gly Lys Lys Ser Phe Ser Asn Leu

    TAG ATT TTC TGA TGT AAG ATT AGT TAA ATC TTT AAC ATA   3225
165 Leu Asn Glu Ser Thr Leu Asn Thr Leu Asp Lys Val Tyr
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FIG 3 (Cont'd) 5/7

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    ATG CCA AGT TAC TTG CTC ACT ATT AAA ACC AAC TTC ATC  3264
152 His Trp Thr Val Gln Glu Ser Asn Phe Gly Val Glu Asp

    CCC CTG ATA TGT AAA ACC AGC TTT TTC AAA AAT ATC TCT  3303
139 Gly Gln Tyr Thr Phe Gly Ala Lys Glu Phe Ile Asp Arg

    TAA TTC AAT ATT ACC ATC TTG AAT TAA CCT ACC TTC ATC  3342
126 Leu Glu Ile Asn Gly Asp Gln Ile Leu Arg Gly Glu Asp

    ATC ATA ATA TTG ATA ATC ATC ATA TGG AAA AAT ATC AAG  3381
113 Asp Tyr Tyr Gln Tyr Asp Asp Tyr Pro Phe Ile Asp Leu

    TTC TAA TAC ATT ATT TTT TTT CGT ATA TTT TTT TAA TTC  3420
100 Glu Leu Val Asn Asn Lys Lys Thr Tyr Lys Lys Leu Glu

    AAT CAA AAA ATG TGC AAG ATA TCT TTC TTC ACT AAA AAT  3459
 87 Ile Leu Phe His Ala Leu Tyr Arg Glu Glu Ser Phe Ile

    AGG TCC ATA ATA AAT ACC CAT ATT CAA GCC ACC AAA TAT  3498
 74 Pro Gly Tyr Tyr Ile Gly Met Asn Leu Gly Gly Phe Ile

    TTT TTG GGT ATA TGT AAG TGC GAC AAC CTT TAT TTC CTC  3537
 61 Lys Gln Thr Tyr Thr Leu Ala Val Val Lys Ile Glu Glu

    CAA ATG AAA AAG AGC AAA ATA TTT AAC ATC AAA ATT TCG  3576
 48 Leu His Phe Leu Ala Phe Tyr Lys Val Asp Phe Asn Arg

    ACT CTT TCT TAA ATT TCC CAT TTC AAT GGT TTG CTC AAA  3615
 35 Ser Lys Arg Leu Asn Gly Met Glu Ile Thr Gln Glu Phe

    AGA ACG TCT TTT CTG AGT ATT TGC AAA TTT TTC AAA AGT  3654
 22 Ser Arg Arg Lys Gln Thr Asn Ala Phe Lys Glu Phe Thr

    AAG TGC ATC GAT TTC TTG AAA TTT CAT ATTTTATTTCCTATA  3696
  9 Leu Ala Asp Ile Glu Gln Phe Lys Met   <Zif cds

TTTCTTCCTAGTAAAATAATAACAATAAATTATAAAACGAATAAATTATAA  3747
AAAATTTTAAGTTTCTTTTCGATTTTGTTACAATAACTTACATTTTAAAAA  3798
CCTTAAAACTAAAAGTTGAAAATTCTATATTAATAGTTTATCATAATATTC  3849
GAGAAGGCGCTTTCTTTTTAAAGATTTATAATACTGTAATTTACTTTAGTA  3900

AAGTTCTTTTTATATGGAGGATAAAAT ATG AAA CGT ATA TTT TTT  3945
                  <ZooA cds   Met Lys Arg Ile Phe Phe

    GCT TTC TTA AGT TTA TGC TTA TTT ATA TTC GGA ACA CAA  3984
  7 Ala Phe Leu Ser Leu Cys Leu Phe Ile Phe Gly Thr Gln

    ACG GTA TCT GCA GCT ACT TAT ACT CGG CCA TTA GAT ACG  4023
 20 Thr Val Ser Ala Ala Thr Tyr Thr Arg Pro Leu Asp Thr

    GGA AAT ATC ACT ACA GGG TTT AAC GGA TAC CCT GGT CAT  4062
 33 Gly Asn Ile Thr Thr Gly Phe Asn Gly Tyr Pro Gly His
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FIG 3 (Cont'd) 6/7

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    GTT GGA GTC GAT TAT GCA GTA CCC GTT GGA ACT CCG GTT  4101
46  Val Gly Val Asp Tyr Ala Val Pro Val Gly Thr Pro Val

    AGA GCA GTT GCA AAT GGT ACA GTC AAA TTT GCA GGT AAT  4140
59  Arg Ala Val Ala Asn Gly Thr Val Lys Phe Ala Gly Asn

    GGG GCT AAT CAC CCA TGG ATG CTT TGG ATG GCT GGA AAC  4179
72  Gly Ala Asn His Pro Trp Met Leu Trp Met Ala Gly Asn

    TGT GTT CTA ATT CAA CAT GCT GAC GGG ATG CAT ACT GGA  4218
85  Cys Val Leu Ile Gln His Ala Asp Gly Met His Thr Gly

    TAT GCA CAC TTA TCA AAA ATT TCA GTT AGC ACA GAT AGT  4257
98  Tyr Ala His Leu Ser Lys Ile Ser Val Ser Thr Asp Ser

    ACA GTT AAA CAA GGA CAA ATC ATA GGT TAT ACT GGT GCC  4296
111 Thr Val Lys Gln Gly Gln Ile Ile Gly Tyr Thr Gly Ala

    ACC GGC CAA GTT ACC GGT CCA CAT TTG CAT TTT GAA ATG  4335
124 Thr Gly Gln Val Thr Gly Pro His Leu His Phe Glu Met

    TTG CCA GCA AAT CCT AAC TGG CAA AAT GGT TTT TCT GGA  4374
137 Leu Pro Ala Asn Pro Asn Trp Gln Asn Gly Phe Ser Gly

    AGA ATA GAT CCA ACC GGA TAC ATC GCT AAT GCC CCT GTA  4413
150 Arg Ile Asp Pro Thr Gly Tyr Ile Ala Asn Ala Pro Val

    TTT AAT GGA ACA ACA CCT ACA GAA CCT ACT ACT CCT ACA  4452
163 Phe Asn Gly Thr Thr Pro Thr Glu Pro Thr Thr Pro Thr

    ACA AAT TTA AAA ATC TAT AAA GTT GAT GAT TTA CAA AAA  4491
176 Thr Asn Leu Lys Ile Tyr Lys Val Asp Asp Leu Gln Lys

    ATT AAT GGT ATT TGG CAA GTA AGA AAT AAC ATA CTT GTA  4530
189 Ile Asn Gly Ile Trp Gln Val Arg Asn Asn Ile Leu Val

    CCA ACT GAT TTC ACA TGG GTT GAT AAT GGA ATT GCA GCA  4569
202 Pro Thr Asp Phe Thr Trp Val Asp Asn Gly Ile Ala Ala

    GAT GAT GTA ATT GAA GTA ACT AGC AAT GGA ACA AGA ACC  4608
215 Asp Asp Val Ile Glu Val Thr Ser Asn Gly Thr Arg Thr

    TCT GAC CAA GTT CTT CAA AAA GGT GGT TAT TTT GTC ATC  4647
228 Ser Asp Gln Val Leu Gln Lys Gly Gly Tyr Phe Val Ile

    AAT CCT AAT AAT GTT AAA AGT GTT GGA ACT CCG ATG AAA  4686
241 Asn Pro Asn Asn Val Lys Ser Val Gly Thr Pro Met Lys

    GGT AGT GGT GGT CTA TCT TGG GCT CAA GTA AAC TTT ACA  4725
254 Gly Ser Gly Gly Leu Ser Trp Ala Gln Val Asn Phe Thr

    ACA GGT GGA AAT GTC TGG TTA AAT ACT ACT AGC AAA GAC  4764
267 Thr Gly Gly Asn Val Trp Leu Asn Thr Thr Ser Lys Asp

    AAC TTA CTT TAC GGA AAA  TAA   TTATTAAAATTTATAGAAAT  4805
280 Asn Leu Leu Tyr Gly Lys [Stop]
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FIG 3 (Cont'd) 7/7

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ATTTAATATAGACTATTAGACGTTCCTTTTTTTGGCTCTTTGTCAACTGT  4855
AGTAGGTAGTTGACAAGCTAACATCTGGAGAGGACCAAATTGGTCTTCTC  4905
TTTTTTCATATTGATAGCGATCAAAATCCGTCTTTTAAAGTTTTCAAAGT  4955
TCCGAAATCCAAAAGCATTGCGCTTGATGACTTTGATGAGATTATTGGTA  5005
GCCTCCAGTTTAGCGTTAGAATAAGGCAAATCCAGTGCGTTCTCAATCTT  5055
GTCTCTGTCCTTCAAAAAGGTCTTGAAAACGGTCTGAAAGAGAGGATTGC  5105
TGCTGTCTATCTGCTCCTCAATCAGGCCGAAAAAGTGGTCAGCTTGCTTT  5155
TCCTGGAAGTGAAAAAGCAGAAGCTGATAAAGGTCATAATGCTTTCTAAG  5205
CTCGTCAGAGTAGCTCAAAAGACGCTCGACCACCTCCTTGTTTGTCAAAT  5255
GCATGCGAAAAGTCGGGCGGTAAAAACGCTTGTCACTGAGTTTGCTGCTA  5305
TCTTGTTGAATCAGCTTCCACTAACGTTTCAAAGCCTTGTATTCGTGGCA  5355
TTTTCGATCAAAAGAGTTCATGATTTGAGTACAGATACGGTTCATGGCAC  5405
GTCCCAAATGTTGCACAATGTGGAAACGATCAAGGACAATCTGAGCGTTT  5455
GGGAAAAGCATCTTAGCCAGGTTGTAATAGGGGGTAAACATATCCATGGT  5505
GATGAGTTTAACGTCGTTTCTGACCTGCCGAGGATATCTCAGGAAGTGGT  5555
TGCGAATGACAGCTTTCGTCCGTCCATCCAAAATAGCGATGATGTTGTTG  5605
GTGTCAAAGTCCTGAGCAATAAAGCTCATCTTGCTCTTCTTAAAGGCATA  5655
TTCATCCCAGGACATATGTTCGGGTAAATAAGTCAAATGAGACTTGAACG  5705
TGAACTCGTTGAGCTTTCGCATGACGGTTGAGGTGGAGATGGATAGTCTA  5755
TCAGCGATAGTGGTCATGGAGACTTTCTCGATGAGAAGCTGGGCCACCTT  5805
CTGTTTGACGATGGTTGGTATTTGATGGTTTTTAGGGACTAGAGAAGTCT  5855
CAGCGACAGTAATTTTTCCGCAAAACTGACATTTGAAACGGCGCTTTTTG  5905
AGGCGAATCAAAGTCTTGTAGCCTGCGCAATCGAGAAAAGGGACCTTGGA  5955
TTCCCGTTGGAAGTCGTACTTGCCCATCTGACTTTGGCAGTTAGGGCAAG  6005
GTGGGGCATCGTAGTCAAGGACAGCTTTCAATTCCTTATGAGTTTTCATG  6055
TCGTGTATTTCTTTGGAGAATGTGATATGAGGGTCTTTAATTCCCAGTAG  6105
TTGTGTGATAACATGTGATTGTTCCATATGAGTCTTTCTAAATGATAGTT  6155
TAGTCGCTTTTCATTATAGGTCATATGGGACTTTTTTGATACTCATAAAG  6205
CCCTATAACCCCTGCAGTGGCCTTACCCACTACGGAAATTATACATATAT  6255
TCTTTATGCTATAATATAAGTCAAAGTACAGCATTGACAAAGGAGACAAA  6305
GATTGAAAATTGTAATAATTGGCTATAGTGGTTCTGGAAAGTCGACTTTA  6355
GCAAATGTTTTAGGTCAACACTACAATTGTGCTGTACTTCATTTAGACAA  6405
AATTCATTTTGCATCAAACTGGCAAGAGCGAACAGTTAGTCAAATGGTCT  6455
CTGATATATCAACATTTATGTCACAAAAACATTGGATTATTGAAGGTAAT  6505
TATTCAAGCTGTCTTTATGAAGAGCGTATGAGAGAGGCTGATCACATTAT  6555
ATATTTTAACTTTAATAGATTTAATTGTTTTTACCGAGCTTTTAAGCGAT  6605
ATTTAAAATATAGGGGACAAACACGTCCTGATATGGCTGAAAACTGTAAT  6655
GAAAAATTTGATGTTGAATTTATGAAATGGATTCTGTTAGACGGACGCTC  6705
AAAAAATAATTTAAATAACTATAAAACAGTTATTAAAACATATCCTCATA  6755
AAATAATCGTTTTAAAAAATCAAAAGCAGTTAATTCATTATATGAATTC   6804
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00171

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 14/315; C07H 21/04; C12N 1/21; A23L 1/00, 1/015; A01N 63/00; A61K 35/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN (Medline, CA): Keyword-zoocin?
ANGIS (BLASTP, FASTA): sequence ID No. 1
STN subsequence search:
MKFQEIDALTFEKFANTQKRRSFEQTIEMGNLRKSRNFDVKYFALFHLEEIKVVALTYTQKIFGGLNMGIY YGPIFSEER/SQSP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	FEMS Microbiology Letters, Volume 163, 1998, S. A. Beatson et al., 'Zoocin A Immunity Factor: A Fem A-like Gene Found in a Group C *Streptococcus*', pages 73-77. (see the whole document)	1-31

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 1999	24 MAR 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer Marie-Anne Fam **MARIE-ANNE FAM** Telephone No.: (02) 6283 2259

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00171

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gene, Volume 189, April 1997, R. S. Simmonds et al., 'Cloning and Sequence Analysis of zooA, a *Streptococcus zooepidemicus* Gene Encoding a Bacteriocin-Like Inhibitory Substance Having a Domain Structure Similar to that of Lysostaphin', pages 255-261. (see in particular pages 256-257, 2.2 'Nucleotide sequence Analysis' and page 259, text relating to figure 2, Genbank Accession Number U50357).	3-8
A	Applied and Environmental Microbiology, Volume 62, 1996, R. S. Simmonds et al., 'Mode of Action of a Lysostaphin-like Bacteriolytic Agent Produced by *Streptococcus zooepidemicus* 4881', pages 4536-4541.	1-31